Deep Brain Stimulation of the Subthalamic Nucleus, Effect on Parkinsonian Motor Symptoms and Experimental Effects with CDNF

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting about 1% of people over 65. The cardinal motor symptoms of PD are mostly due to reduced dopaminergic signaling in the nigrostriatal system and can be effectively treated with dopaminergic medication. However, the most effective treatment, levodopa (L-DOPA), leads to dyskinesias or motor fluctuations. In selected patients, this can be treated with deep brain stimulation of the subthalamic nucleus (STN DBS). Despite extensive studies, the exact mechanisms and optimal targeting of electrodes of STN DBS remain incompletely understood. The current treatments offer only symptomatic relief and no effective neuroprotective therapies are currently available, although neurotrophic factors (NTF) such as CDNF have been promising, especially in animal models.

Our aims were to provide a better description of the antiparkinsonian effects of STN DBS both in an experimental rat model and in clinical STN DBS; and to study possible therapeutically favorable interactions of experimental STN DBS and CDNF.

Our approach was to use a retrospective clinical study to examine STN DBS electrode locations and reductions in dopaminergic medication and PD symptoms. A significant reduction was observed in both medication and PD symptoms, and more ventrally located electrodes seemed to be more effective, contradicting some earlier studies.

We studied the effects of STN DBS in a medial forebrain bundle 6-hydroxydopamine (MFB 6-OHDA) rat model that shows a severe dopaminergic depletion similar to that seen in advanced PD. Stimulation-induced dyskinesias were increased with higher stimulation amplitudes, and these dyskinesias also correlated with an improvement in front-limb use defect. These findings add to our understanding of this relationship and the dose-response of the antiakinetic effect of STN DBS.

The combination of STN DBS and CDNF was similarly studied in the MFB 6-OHDA rat model. In particular, the STN lesion, used as a model of chronic STN DBS, had an additive effect with CDNF in reducing the behavioral effects of 6-OHDA hemiparkinsonism, seen both in apomorphine-induced rotations and in the cylinder test for front limb use. These findings indicate that a combination of CDNF and experimental STN DBS has an additive effect in ameliorating the motor deficits of PD. STN DBS and NTF treatments earlier in the course of PD have been suggested as a way to improve their effectiveness. The data presented here provide the first experimental evidence that the effectiveness of STN DBS and NTFs might be improved by administering them simultaneously.

TIIVISTELMÄ

Parkinsonin tauti on Alzheimerin taudin jälkeen toiseksi yleisin rappeuttava aivosairaus, josta kärsii noin 1% yli 65 vuotiaista ihmisistä. Parkinsonin taudin (PD) keskeiset liikehäiriöt johtuvat aivojen nigrostriataalisen dopamiinivälittäjäainejärjestelmän toiminnan heikentymisestä. Näitä oireita voidaan tehokkaasti hoitaa levodopalääkityksellä, joka kuitenkin johtaa suurella osalla potilaista aikanaan hallitsemattomiin pakkoliikkeisiin tai tilanvaihteluihin. Osalla potilaista näitä oireita voidaan tehokkaasti hoitaa subtalaamisen tumakkeen syväaivostimulaatiolla (STN DBS). Huolimatta aiheen pitkäkestoisesta ja laaja-alaisesta tutkimuksesta STN DBS:n tarkkaa vaikutusmekanismia ja optimaalista stimulaatiokohtaa tarkasti kohdealueella ei täysin tunneta. Nykyiset hoidot eivät vaikuta PD:n etenemiseen vaikkakin hermokasvutekijät, kuten CDNF, ovat olleet lupaavia PD:n eläinmalleissa.

Tavoitteenamme oli kuvata tarkemmin STN DBS:n vastetta sekä kliinisessä PD:ssä että rottaeläinkoemallissa sekä tutkia STN DBS:n ja CDNF:n mahdollisia suotuisia yhteisvaikutuksia koe-eläinmallissa.

Kliinisessä tutkimuksessa määritimme asetettujen elektrodien tarkan sijainnin sekä STN DBS –hoidon vaikutuksen potilaiden käyttämän dopamiinilääkitysten määrään sekä PD:n oireisiin. PD:n oireet ja potilaiden dopamiinilääkityksen tarve vähenee merkittävästi. Ventraalisemmin sijaitsevat elektrodit vaikuttivat johtavan hieman parempiin hoitotuloksiin, mikä on osin ristiriidassa aiempien tutkimustulosten kanssa.

Eläintutkimuksessa aiheutimme rotille toispuolisen pitkälle edennyttä PD:tä vastaavan tilan ruiskuttamalla dopamiiniradastoon 6-OHDA:a. Stimulaatiovirran asteittainen nostaminen aiheutti lisääntyviä pakkoliikkeitä sekä samanaikaisen toispuolisen PD:n liikeoireen korjaantumisen. Tulokset lisäävät tämän yhteyden ymmärrystä sekä mahdollistavat eläinkokeiden menetelmällisen kehittämisen.

Tutkimme STN DBS:n ja aivojen substantia nigraan ruiskutetun CDNF:n vaikutusta yhteisvaikutusta samassa 6-OHDA eläinmallissa. Etenkin kroonisen stimulaation mallina käytetyllä STN leesiolla oli myönteinen yhteisvaikutus CDNF:n kanssa PD:n eläinmallin oireiden korjaamisessa, kun kumpikaan hoito yksinään ei ollut tehokas. Tämä yhteisvaikutus todettiin kahdella käyttäytymiskokeella, mutta selviä biokemiallisia todisteita yhteisvaikutuksesta ei ollut. Tutkimuksessamme osoitetaan ensimmäistä kertaa kokeellisesti hermokasvutekijän ja STN DBS suotuisa yhteisvaikutus. CDNF hoidon heikko teho yksinään tukee aiempia tutkimustuloksia, joiden perusteella kasvutekijähoitoja on ehdotettu kokeiltavaksi myös PD:n varhaisemmassa vaiheessa. Mikäli suotuisasta yhteisvaikutuksesta saadaan jatkossa lisänäyttöä, voidaan tämän tutkimuksen tulosten perusteella mahdollisesti kehittää uusi, tehokkaampi, yhdistelmähoito PD:ssa.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAV	adeno associated virus
AChEI	Acetylcholinesterase inhibitors
BBB	Blood brain barrier
CNS	Central nervous system
COMT	Cathecol-O-methyl transferase
CSTS	Cortico-striato-thalamocortical system
CT	Computer tomography
D1	Dopamine receptor type 1
D2	Dopamine receptor type 2
DA	Dopamine
DAT	Dopamine transporter
DBS	Deep Brain stimulation
BMT	Best medical therapy
GPe	Globus pallidus externa
GPi	Globus pallidus interna
HFS	High frequency stimulation
H&Y	Hoehn and Yahr scale
IHC	Immunohistochemistry
IPG	Implantable pulse generator
LB	Lewy body
L-DOPA	Levodopa
LID	Levodopa-induced dyskinesia
LN	Lewy neurite
MFB	Median forebrain bundle
MSN	Medium spiny neuron
MPTP	(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)
MRI	Magnetic resonance imaging
NE	Norepinephrine
NMDA	glutamatergic N-methyl-D-aspartate receptor
NTF	Neurotrophic growth factor
PNS	Peripheral nervous system
RCT	Randomized clinical trial
ROS	Reactive oxygen species
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
sPD	Sporadic Parkinson’s disease
STN	Subthalamic nucleus
STNL	Subthalamic nucleus lesion
PD	Parkinson’s disease
QoL	Quality of life
TH	Tyrosine hydroxylase
UPRDS	Unified Parkinson’s disease rating scale
VTA	Ventral tegmental area

LIST OF ORIGINAL PUBLICATIONS

I. Huotarinen A, Leino S, Tuominen R, Laakso, A Evaluation of subthalamic stimulation in rat: choosing stimulation current and defining a good hit. *Submitted to MethodsX*

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III. Koivu M, Huotarinen A, Scheperjans F, Laakso A, Kisaari R, Pekkonen E, Motor outcome and electrode location in deep brain stimulation in Parkinson's disease. *Brain and Behavior*. 2018 Jul;8(7):e01003

Publication III will be used in the thesis of Maija Koivu MD.

1 INTRODUCTION

Parkinson's disease (PD) was first described 200 years ago, and since then, the understanding of the pathological mechanisms and the development of treatments have been subjects of considerable research. The treatment of PD has been revolutionized twice: first by the pharmacological dopaminergic substitution by levodopa (L-DOPA)[1] and later by deep brain stimulation of the subthalamic nucleus (STN DBS).[2] Although L-DOPA is very effective at reversing the cardinal motor symptoms of PD, it leads almost inevitably to L-DOPA–induced dyskinesias or motor fluctuations.[3] Prior to the advent of L-DOPA, stereotactic surgery for movement disorders had been introduced, and lesional surgeries provided substantial improvement in the 1950s despite the primitive imaging possibilities available at the time.[2,4] However, the introduction of L-DOPA in 1968 brought stereotactic surgery to almost a complete stop.[4] In 1987, Benabid et al. described the use of high frequency stimulation as a reversible lesion analogue[5] and paved the way to clinical introduction of STN DBS as a PD treatment and subsequent popularization of the procedure.[6]

STN DBS is a clinically effective treatment that has been extensively studied both clinically and in experimental animal models, but the precise mechanism of its effect remains enigmatous.[7] Even the exact location of optimal electric stimulation within STN, or even outside the STN, is subject to continuing debate.[8]

However, the available treatments offer only symptomatic relief, and no current treatment can slow or reverse the progression of PD.[9,10] Several possible treatments have been studied, and although many have been successful in experimental models, none has been uniformly effective in treatment of clinical PD. One reason for this non-success might be the fact that the dopaminergic degeneration is already substantial by the time of onset of PD symptoms, and by 5 years after diagnosis, the dopaminergic system is practically devoid of dopaminergic neurites.[11] Both potential neuroprotective treatment therapies and STN DBS are commonly offered to patients with over 5 years of disease duration[12], highlighting the requirement to develop neuroprotective treatments that would work in clinically realistic circumstances.[13]

One promising class of neuroprotective agents are the neurotrophic growth factors (NTFs)[14], which have shown positive results in post-hoc analysis in a subset of patients with shorter duration of disease.[15] Because the treatment results with NTFs have been quite modest, NTFs are not expected to function as monotherapies in PD, providing a rationale to study the interactions of NTFs with symptomatically effective therapies.

2 REVIEW OF THE LITERATURE

2.1 Parkinson's disease

2.1.1 Clinical and historical overview of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease globally, affecting approximately 1% of people over 65[16-18] with the average prevalence increasing with older age. It was first described in Western medical literature in an essay "On the shaking palsy" by James Parkinson in 1817 (recently republished [19]). Parkinson's disease was also described in the ancient Ayurvedic literature[20] and by the Roman physician Galen,[21] among others.[22] PD was further distinguished as a separate clinical entity by Charcot and his students, who also described atypical variants of PD and early pharmacological treatment efforts with anticholinergic agents and ergot alkaloids.[22] Anticholinergic agents remained the mainstay of PD treatment efforts until the advent of levodopa (L-DOPA) treatment in 1961.[1,23] In the preceding years, dopamine had been localized in the brain, especially in the striatum, and levodopa had been found to be the precursor of dopamine.[24] Another finding was that reserpine produced motor symptoms similar to those seen Parkinson's disease and these symptoms were reversed by L-DOPA.[23] Subsequently, L-DOPA and dopamine agonists were established as effective therapies for PD, although the common occurrence of L-DOPA related side effects, such as dyskinesias and motor fluctuations, was soon discovered.[23,25] The efficacy of L-DOPA drug regimens and control of L-DOPA side effects further improved with co-administration of dopa-decarboxylase inhibitors, such as carbidopa[25,26] and, later, cathecol-O-methyl transferase (COMT) inhibitors, such as entacapone.[27,28]

The general hallmark of PD is the slow progression of parkinsonian motor symptoms, especially slowness of movements (bradykinesia), rigidity, and rest tremor,[29] which are the cardinal symptoms of PD. In addition to these cardinal symptoms, various non-motor symptoms have been described, including hyposmia, sleep disorders, depression, and constipation. These non-motor symptoms can precede the onset of motor symptoms by several years.[30,31] During the prodromal phase, the major neurobiological correlate of progressing PD is the advancing degeneration of striatonigral dopaminergic neurons,[11,29] and motor symptom onset is thought classically to occur when dopaminergic degeneration reaches 50–60% [32,33]. With advancing disease, the motor and non-motor symptoms gradually worsen, leading to greater impairment and the eventual appearance of falls, postural instability, dysphagia, and cognitive decline leading ultimately to Parkinson-related dementia in many patients.[31] Although many of these symptoms are well controlled by L-DOPA, MAO-B, and dopamine agonists, eventually many patients develop severe medication-related side effects.[34,35]

The diagnosis of PD is made based on the presence of the cardinal motor symptoms.[36] However, the accuracy of initial clinical diagnosis is not very good.[37-39] Along with disease progression, some patients develop additional symptoms that belong to atypical parkinsonian syndromes, which generally have a worse prognosis than Parkinson's disease itself. Rare cases also occur where there is a clear exposure to a chemical agent known to cause PD, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)[40] and the pesticide rotenone.[41] Despite the rarity of these toxic forms of PD, they provide some support for the validity of commonly used neurotoxin animal models. The disease cases

where no chemical exposure is evident, no familial history of PD exists, and no signs of atypical parkinsonian syndromes develop are generally referred to as sporadic Parkinson's disease (sPD)[42], although a complex interplay probably occurs between environmental and genetic factors.[43] The inaccuracy of the initial diagnosis, and especially the relative inability to exclude the possibility of atypical Parkinsonian syndromes, is one the main reasons why invasive and experimental PD treatments often require a five-year follow up after the diagnosis. Some researchers have argued that this has led to suboptimal results in trials of potential neuroprotective and neurorestorative treatments.[13]

PD is a progressive disease and the progression and the severity of the disease can be monitored with a scale originally published by Hoehn and Yahr.[44] The time needed to reach the more advanced disease states[45] can vary considerably.[46] The life expectancy of PD patients is reduced[16] (mortality hazard ratio 1.5–2.7) and the risk of death is increased in patients who develop Parkinson-related dementia.[47] Although dopaminergic drugs provide good symptomatic control, they do not slow the progression of the disease. Various treatments have been studied in order to modify disease progression but have had disappointing results.[48] Among these therapies are the use of various neurotrophic growth factors (NTFs), which have shown successful results in animal studies but have generally failed clinical blinded trials.[49] The quality of life (QoL) in Parkinson's disease is decreased, especially in the later stages[50] where patients also become dependent on outside care. The non-motor symptoms, in particular, lead to a decreased quality of life.[51] Some non-motor symptoms also fail to respond well to dopaminergic or other pharmacological treatments, especially in the later stages of PD.[52]

The cost of the disease burden is high in PD patients: in 2010, direct costs per patient averaged $12,805 and indirect costs $10,046 in the US[53] and 5626€ direct and 5526€ indirect in Europe.[54]. This amounts to a disease cost for PD of over $14,4 billion yearly in the US alone and €13,9 billion in Europe. The current aging of the population is leading to overall increases in these costs, and neuroprotective therapies are now being promoted as potential ways to both increase the quality of life and decrease the economic cost of PD.[55]

2.1.2 The dopaminergic system and the basal ganglia

The dopaminergic system is one of the monoaminergic neuromodulatory systems in the brain,[56] and it exerts wide tonic control on various functions in the central nervous system (CNS). The majority of dopamine neurons belong to either nigrostriatal or mesolimbic/-cortical pathways. The soma of nigrostriatal neurons are located in the substantia nigra pars compacta (SNpc) and they project axons to the striatum. The soma of the mesolimbic/-cortical dopaminergic neurons are located in the ventral tegmental area (VTA) and they project their axons to the limbic system and to the cortex. Dopamine and other catecholamines act as modulators of neural signaling rather than as synaptic transmitters and they regulate various important behaviors.[57]

Understanding the function of dopamine in the brain also requires that the anatomy and physiology of the dopaminergic system, basal ganglia, and cortico-striato-thalamocortical systems (CSTS) to be described. The CSTS comprises complex [58-59] and parallel networks [60] that contribute to the regulation of a wide variety of functions and behaviors, ranging from motor functions to limbic and associative functions. The parallel networks have some shared and some distinct

components.[60] The following description focuses mainly on the motor CSTC, which is of greatest importance in understanding the cardinal motor symptoms of PD.

A widespread glutamatergic excitatory projection extends from the cortex to the striatum, where cortical fibers connect with striatal medium spiny neurons (MSN) that receive either excitatory (D1, direct pathway) or inhibitory (D2, indirect pathway) dopaminergic input from the substantia nigra pars compacta (SNc).[61] The main output pathway of the striatum is the globus pallidus interna (GPi) and substantia nigra pars reticulata (SNr) for the direct pathway and the globus pallidus externa (GPe) for the indirect pathway. The main output of GPe is to the subthalamic nucleus, and the GPe receives reciprocal output from the STN; projections also extend to the SNr and GPi. STN also has output to the SNr/GPi, where the direct and the indirect pathways converge. The SNr/GPi have their main outputs to the thalamus, which in turn projects back to both the striatum and the cortex to create the CSTC loop (Figure 1). This canonical model of the CSTC networks have been challenged to provide a better explanation of some phenomena, but for understanding the overall structure and function the canonical model has still held its value.[59] Major alterations and dysfunctions in any part of this network can lead to disruption in the overall function of the CSTC network. However, this also provides the opportunity to treat a dysfunctional CSTC loop by delivering treatments that affect only anatomically isolated components of the loop; this is the basis for the stereotactic functional neurosurgical treatments described in detail later (chapter 2.2).

Neural signaling in the CSTC has been studied extensively at both the cellular and intracellular levels in different anatomical locations. The signaling in the main dopaminergic end organ—the striatum—has

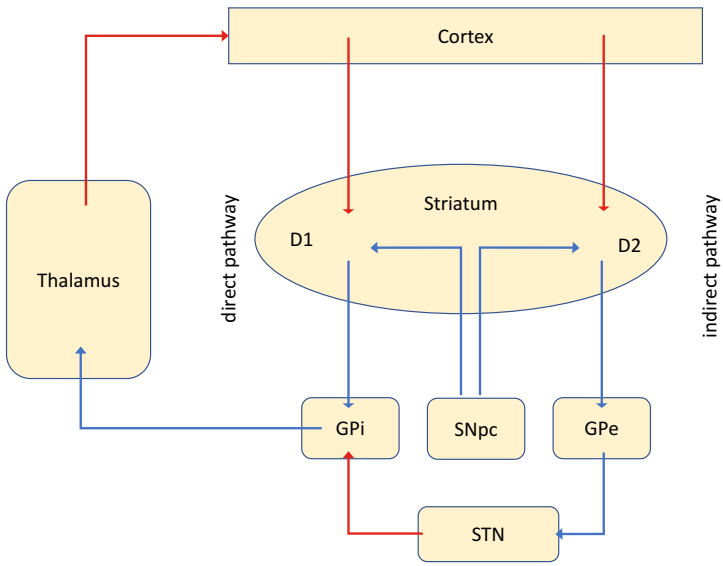

Figure 1 The canonical model of CSTC network organization.
D1 = D1-receptor positive medium spiny neurons, D2 = D2-receptor positive medium spiny neurons, GPe = globus pallidus externa, GPi = globus pallidus interna, STN = subthalamic nucleus, Excitatory connections in red and inhibitory connections in blue. *Adapted and modified from Lanciego 2012*[59]

been described extensively [62,63]. The medium spiny neurons of the striatum belong to either the D1-type population (direct pathway, D1 & D5) and D2-type population (indirect pathway D2,D3,D4), according the type of receptors expressed on the cell membrane of the dendrites. In both of these populations, the dopamine receptors transmit the extracellular signal to a G-protein (Gαs, Gαolf) coupled intracellular signaling system. In the D1 positive MSNs, dopaminergic signaling leads to an excitatory increase in adenylyl cyclase (AC, cAMP) activity and, conversely, in the D2 positive MSNs, dopamine signaling leads to a G-protein (Gαi/o) coupled decrease in the AC activity.[64] Further downstream, D2-signalling is mediated by β-arrestin 2, PP2A, and Akt containing complexes.[65] Additionally, inhibitory D2-receptors are located also presynaptically in the axon terminals, where they serve as autoreceptors. In the dopamine depleted striatum, a compensatory increase occurs in the sensitivity of the post-synaptic signaling, which is termed supersensitivity. This phenomenon is used experimentally in hemiparkinsonian animal models, where postsynaptically active dopaminergic drugs can be used to induce higher activation of the lesioned side. This causes rotational behavior that, in turn, can be used as a crude measure of the loss of dopaminergic tone.[66] In an animal model, supersensitivity-related rotations require from week[67] to several weeks to develop.[68]

2.1.3 Pathobiological basis of Parkinson's disease

Although the exact pathological process behind sporadic PD is not clear, it is now generally accepted that accumulation of α-synuclein[69] and the formation of Lewy bodies (LB) and Lewy neurites (LN) in the CNS (central nervous system) are among the central pathological processes.[70] The dopaminergic neurons of the midbrain are particularly sensitive to the effects of α-synuclein accumulation and the ensuing degeneration. These changes are widespread in PD, especially in the advanced stages, and are thought to develop in an ascending fashion, starting from the caudal brainstem and eventually reaching cortical structures.[70] However, this ascending theory has been recently challenged by functional threshold theory, which provides a better explanation of the symptoms derived from peripheral nervous system (PNS) during the sometimes prolonged prodromal phase of the disease.[71] The functional threshold theory claims that the neuronal degradation induced by α-synuclein occurs in parallel in the PNS and CNS and that the PNS symptoms precede the CNS dopaminergic symptoms because the dopaminergic system has a greater redundancy and so it tolerates a higher degree of degeneration before symptoms appear. The onset of motor symptoms correlates with a 50–60% loss of dopaminergic neurons,[32] and the loss of dopaminergic terminals in the striatum is reported to continue for at least 4 years post-diagnosis to be nearly complete in a post-mortem study.[11]

The etiology of Parkinson's disease can be divided into sporadic and genetic/familial forms, with the familial form comprising less than 10% of cases.[72] The pathological mechanism is thought to result from some genetic causation, environmental factor, or their combination.[72,73] Chemical compounds, such as the pesticide rotenone and MPTP, have well-known linkages to PD and PD-like syndromes.[73] Genetic and familial cases have been shown to relate to various autosomal genes, such as SNCA, LRRK2, Parkin, and PINK1.[74] The etiology of the more common sporadic PD has been a subject of more controversy. However, increased oxidative stress and mitochondrial damage in dopaminergic neurons has been suggested as a common factor in different forms of clinical

PD and animal models of PD.[72] Currently, one of the most attractive theories is the accumulation of pathological forms of α-synuclein in the gastric neurons related to gut microbiota.[75] The α-synuclein-related changes in the gastric neurons are thought to be propagated to the CNS supposedly by ascending along the vagus nerve. The now classical model is the Braak theory,[70] which describes the ascending propagation of α-synuclein pathology (LB and LN) starting from the caudal brainstem and eventually affecting the whole brain, including the cortex, at the most advanced stages.

The effects of dopaminergic cell loss on the basal ganglia signaling has been described in detail at both the cellular level[76,77] and brain network level, especially for the functionally most important CSTC pathways. [78,58,79] In Parkinson's disease, the dopaminergic output in striatonigral neurons is decreased and this leads to compensatory changes in the motor CSTC. The main effect of decreased dopaminergic tone in the striatum has been shown to be a shift in balance toward increased activity in the so-called indirect pathway compared to the direct pathway.[80] The overall effect of the indirect pathway is inhibitory, whereas the overall effect of the direct pathway is excitatory. The inhibitory effects of the indirect pathway are mediated by the increased activity of the subthalamic nucleus (STN) in response to dopamine depletion.[78,80,81].

The STN has been suggested to function as a general inhibitory brake in the brain by decreasing thalamocortical excitation through increases in the inhibitory drive of the GPi and SNr to the thalamus.[82-84] L-DOPA corrects some of the related pathological changes, but intermittent L-DOPA administration in particular can lead to overactivity of the thalamocortical pathway and the appearance of L-DOPA-induced dyskinesias (LID) seen clinically, especially in the more advanced stages of PD[85]. The mechanisms of L-DOPA-induced dyskinesias have been of considerable research interest both clinically and pre-clinically.[85,86] One of the main phenomena related to LIDs is the hyperactivation of the D1-positive MSNs of the direct pathway, which is reflected by an increase in DARPP-32(thr34) phosphorylation after chronic and intermittent administration of L-DOPA, resulting in a concurrent increase in LIDs.[87] The severity of LIDs in experimental rodent models has been assessed on a rating scale (0–4) based on the duration and severity of the involuntary dyskinetic movements.[88] The dyskinesias are further divided into orofacial, axial, front limb, and locomotive subtypes.

Overall, the pathobiology of PD can be crudely divided into pathological changes related to or causing the dopaminergic cell loss and the progression of the disease and the changes related to the effects of decreased dopaminergic signaling, especially in the striatum, and the mechanisms of symptom formation in PD.

2.1.4 Experimental models of PD

The study of pathological mechanisms and treatments of PD requires the use of experimental animal models. Neurotoxin-induced rodent models have been the mainstay in PD animal research[89], although a number of non-human primate studies have been conducted.[90] Basic research has also been conducted on zebra fish[91] and drosophila,[92] among other species.

The most commonly used animal models are toxin models (6-hydroxydopamine: 6-OHDA and MPTP) that produce a rapid dopaminergic cell death and dopamine depletion.[93] Both 6-OHDA and MPTP are known to aggregate in dopaminergic cells, where they cause cell death by inducing the formation of reactive oxygen species (ROS)

that generate oxidative stress similar to clinical PD but without inducing LB formation.[93] 6-OHDA does not cross the blood brain barrier (BBB) and has to be injected directly to the brain, whereas MPTP readily crosses the BBB and can be administered systematically. Although the use of 6-OHDA means that experimental stereotactic surgery is needed to produce the dopaminergic lesion, 6-OHDA is considered safer for the experimenter. One of the benefits of 6-OHDA is the possibility to produce a unilateral model of parkinsonism, which provides the opportunity to compare the lesioned side to the contralateral unlesioned side and to use pharmacologically induced rotations to measure the depth of the lesion.[94] Unilateral models also have less effect on animal welfare, even when a near total depletion of dopamine is produced. [95] These toxin models produce a reliable dopaminergic lesion, but they do not reflect the pathological mechanisms seen in clinical sporadic PD. However, both 6-OHDA and MPTP produce ROS, which cause mitochondrial damage; consequently, in part, they mimic some aspects of the neuronal damage mechanisms in PD.

Adeno-associated virus (AAV) vectors have also been used to induce overexpression of alpha-synuclein to produce animal models that can better reproduce the proposed pathological mechanism of dopaminergic cell death.[96,97] Furthermore, in these studies, the speed of the dopaminergic lesion is much more rapid than is observed in clinical PD. Virus vector α-synuclein models are one solution to overcome this problem. Their biological mechanism is much closer to PD[98,99], but they might lack predictability, ease of use, and the ability to tailor the model severity to the extent possible with toxin models. The α-synuclein virus vector models also sometimes show synucleopathy without dopaminergic degeneration and behavioral defects.[98] Genetic models[100] are mostly in mice, which could be a limitation in some experimental settings.

Experimentally induced loss of dopaminergic tone replicates some of the stereotypical motor deficits that are seen in clinical PD. [101] This is especially the case for the unilateral lesioning that is often employed, which provides an opportunity to use the asymmetry in motor behavior as a measure of the loss dopamine tone. Similarly, potential therapies can be tested by measuring the reversal of this asymmetry. One of the most commonly used tests is the rotation test, where dopaminergic drugs are given to produce asymmetric striatal activation. The asymmetrical striatal activation produces rotational behavior that can be measured by placing rats in a bowl attached to a harness connected to a rotation measurement system; this produces quantitative data of the asymmetry relatively easily and objectively. [102] Amphetamine, which releases dopamine from striatal terminals, can be used to stimulate the non-lesioned side over the lesioned side, whereas direct dopamine agonists, such as apomorphine, can stimulate the lesioned side over the non-lesioned because of the supersensitivity; the result is contraversive rotations relative to the lesion. [103] Amphetamine-induced ipsiversive rotations develop rapidly after the dopamine depletion because no development of supersensitivity is needed. Amphetamine-induced rotation tests are sensitive for dopamine depletions of 50% or more. Apomorphine-induced rotation tests are sensitive for deeper dopamine depletions of approximately 90%, [104]. Apomorphine-induced rotations can also be seen with less severe dopamine depletion, depending on the dose of apomorphine[105] and the injection site of 6-OHDA[66].

Some of the drawbacks of the rotational test are that pharmacological stimulation is needed to produce a measurable change in

behavior, that this behavior is not a normal behavior exhibited naturally by the rats, and that the results are not directly translatable to results in clinical PD. [95] Other behavioral tests that do not rely on pharmacologic stimulation are the cylinder test[106] and the staircase test,[107] among others; these tests measure different aspects of motor behavior. The cylinder test measures front limb activity by placing the rat in a transparent cylinder and counting the number of front limb touches with the vertical wall during exploration and comparing the use of front limbs as a measure of asymmetry. The cylinder test relies on rat exploratory activity, which might be affected by repetitive application of the cylinder test or disturbances in the experiment room. [106] The cylinder test, however, does not require training of the rat before testing. The staircase test measures skilled front limb use by measuring the number of food pellets that the rat is able to pick with each front limb. The drawbacks of the staircase test are that the rat needs training before testing and must be fasted before individual training or testing sessions. Although these tests provide experimental measure of motor deficits related to dopamine depletion, they do not necessarily replicate the more complex situation in clinical PD. Overall, multiple behavioral tests can be employed in experimental testing to produce more reliable results. [89]

2.1.5 Treatment strategies for PD

The available treatments for PD can provide effective symptom control, especially for the motor symptoms, but no therapies to alter the disease course are yet available.[108] The current treatment strategies for PD depend on the age of the patient, the severity of the symptoms, and the course of the disease.[109,110] The most effective pharmacological treatment of the motor symptoms of PD are orally administered dopaminergic drugs, which can be either dopamine agonists, MAO-B inhibitors, or the more potent L-DOPA. In patients with mild to moderate motor symptoms and age under 60 years, treatment can be started with dopamine agonists or MAO-B instead of L-DOPA[111,112], or even non-dopaminergic drugs such as acetylcholinesterase inhibitors or the NMDA antagonist amantadine.[113] Dopamine agonists or L-DOPA are usually offered to patients with more severe motor symptoms and impairment of activities of daily life. L-DOPA is combined with drugs that inhibit the peripheral (DOPA decarboxylase inhibitors, carbidopa, and benserazide) and the central (CNS) (COMT inhibitor, entacapone, or opicapone) metabolism of dopamine. L-DOPA can cause intolerable dyskinesias and motor fluctuations [114] even a few months after starting this medication,[115] thereby limiting its long-term effectiveness. Attempts can be made to manage the motor fluctuations medically by adding adjunctive medications, such as dopamine agonists, MAO-B inhibitors, and amantadine, or by altering the L-DOPA dosing regimen.[116] Apomorphine, a strong D1- and D2-agonist, is an option for some patients with motor fluctuations.[117,118]

Patients who develop severe dyskinesias or motor fluctuations but retain the L-DOPA treatment effect and do not develop severe cognitive or psychiatric comorbidities are candidates for invasive treatments of PD, such as deep brain stimulation (DBS) and L-DOPA infusion.[116,117] However, the appearance of additional clinical features can sometimes suggest a diagnosis of an atypical parkinsonian syndrome[36 38], where invasive treatments are not generally suitable.

Various disease course altering therapies have been trialed in PD. The effect on the disease course can be divided to neuroprotection, slowing or stopping the degenerative processes, and neurorestoration by reversal of the effects of

degeneration (e.g., by activating regenerative processes). [119] Currently, none has been effective in clinical controlled trials.[48] However, trials are ongoing in the search for effective disease-course altering therapies.[120] So far, the sole aim of treatments for PD is symptom control—and the avoidance of side effects of the treatment.

2.2 Deep brain stimulation

2.2.1 History of stereotactic and functional surgery and Deep Brain Stimulation

The English pioneer of neurosurgery Sir Victor Horsley and the mathematician-surgeon Robert Clarke are commonly acknowledged as the first to have introduced the concept of stereotaxis. In 1908,[121] they presented an apparatus that used a three-dimensional Cartesian coordinate system to introduce probes and needles accurately into targets deep in the brains of experimental animals.[122,123]. They also described the use of electrical currents to produce a controlled and local lesion in the brain and reported their results of electrolytic lesions in the monkey dentate nucleus of the cerebellum using bony landmarks to define a 3-dimensional coordinate system. In 1947, Spiegel and Wycis were the first to report the use of stereotaxy in the human brain, calling their technique "stereoencephalotomy."[124] Instead of bony landmarks of the skull, they used intracerebral landmarks acquired by ventriculography, thereby providing relatively accurate navigation around the third cerebral ventricle. Their procedure originally required the use of pneumoencephalography[125]—the injection of air into the intrathecal space and eventually to the cerebral ventricles to provide contrast to visualize the anatomical landmarks. The technique for lesioning was further enhanced by improvements in thermal radiofrequency lesioning[126,127,128] and intraoperative stimulation for better localization of neuroanatomic targets. [129,130]

Even though there were no frames available commercially, the early years after the introduction of human stereotaxis were an especially fruitful period of both technical innovation and advancements in clinical neuroscience. [131] The introduction of the center of arc principle by Leksell[132] had a particularly strong influence on the future design of later commercially available frames. Some of the other influential frame designs were the Talaraich frame and the Todd-Wells apparatus (later developed to become the Cosman-Roberts-Wells frame).[4]

Prior to the introduction of proper stereotactic systems, various other methods were developed to provide relatively accurate strategies for deep intracerebral lesioning. The most famous example is perhaps the standard leucotomy described in 1935 by the Portuguese neurosurgeon Almeida Lima as a method for limbic leucotomy that later became popularized as frontal lobotomy.[133,134] Although the methods were comparatively accurate for the time, none of these methods came even close to the accuracy and precision of the actual stereotactic systems. Sadly, the methods for lobotomy became even less precise while its use was popularized, and it was applied irresponsibly.[135]

The first patients treated with stereotactic procedures were movement disorder patients[124]. However, the surgery for Parkinson's disease predates the introduction of human stereotaxis. A wide variety of surgical techniques and targets were trialed (including corticotomy[136,137] and posterolateral cordotomy[138]) and early attempts were also made to target the extrapyramidal system for symptom relief.[139] The safety of these surgeries was appalling, with unacceptably high mortality rates. Together with advancement of clinical and pre-clinical neuroscience, these early attempts provided potential targets for later

functional stereotactic procedures. Walker pedunculotomy[140], used for the relief of involuntary movements in PD, later proved especially important for the development of stereotactic targets because of an accidental tearing and subsequent ligation of the anterior choroidal artery. This was found to produce good symptom relief in PD and the ligation of anterior choroidal artery was advocated for a short period by Cooper before he introduced freehand techniques of chemopallidectomy and chemothalamotomy.[141]

During the formative years of stereotactic and functional surgery, many targets were identified, such as the ventrolateral thalamus for tremor lesioning and the globus pallidus and substantia nigra for hemiballismus. The description of thalamic nuclei by Hassler led to a more precise definition of thalamic targets[142] and the Vim nucleus of the thalamus gained popularity as an optimal target for tremor. [143-145] Pallidotomy later evolved to posteroventral pallidotomy, although pallidotomy was thought not to produce benefit on the akinetic symptoms. This was despite reports by Svennilson and Leksell who described improvement in bradykinesia and rigidity with a lesion in the ventral posterior pallidum. [146] Spiegel and Wycis later introduced subthalamic lesioning of the fields of Forel (campus Foreli), which they named campotomy, [147] as a technique with direct effects on the motor symptoms of PD. [148] The first meeting of the International Society of Research in Stereoencephalotomy (predecessor of the World Society for Stereotactic and Functional Neurosurgery) was held in 1966. By 1969, an estimated 37,000 stereotactic operations had been reported[149] with an estimated 25,000 PD patients having undergone operations.[150]

The introduction of L-DOPA in 1968 reduced the number of stereotactic operations for PD to almost zero worldwide, with an ensuing decline in movement disorder surgery in general.[143,150,151] Some of the most important landmark publications in lesional stereotactic surgery for movement disorders are summarized in Table 1.
The resurrection of movement disorder surgery is often attributed to Finnish neurosurgeon Lauri Laitinen, and the description of refined pallidotomy in 1992 is one of the most cited papers in movement disorder surgery.[156] By the late 1980s, the

Table 1 Historical landmarks of lesional movement disorder surgery. Adapted and modified from Okun[152].

Horsley et al. 1909[136]	Motor cortex excision for athetosis
Bucy et al. 1939[137]	Motor cortex excision for PD
Meyers et al. 1942[139]	Open basal ganglia surgery
Spiegel et al. 1946[124]	Stereotactic surgery
Cooper 1953[153]	Anterior choroidal artery ligation
Cooper 1954[154]	Chemopallidectomy and chemothalamectomy
Hassler 1955[155]	Thalamotomies for tremor
Svennilson, Leksell 1960[146]	Posteroventral pallidotomy
Spiegel and Wycis 1962[147]	Campotomy
Laitinen 1992[156]	Pallidotomy revival
Gill 2003[157] Alvarez 2001[158]	Subthalamotomy for PD

debilitating side effects of L-DOPA, such as dyskinesias (LID) and motor fluctuations ("on-off" phenomenon of L-DOPA effect), were indisputable.[23] Posteroventral pallidotomy, as described by Laitinen, provided effective reduction in LID, in addition to symptom reduction of the core symptoms of PD[159], although this was later contested.[160] During the inactive years of movement disorder surgery, the ability to locate the anatomical targets with microelectrode recording had also improved,[161-163] as had the use of computed tomography (CT) and magnetic resonance imaging (MRI) for better visualization of the anatomy.[151] The result was that pallidotomy started to regain popularity and the number of movement disorder surgeries increased worldwide.[143] Pallidotomy could be used as a unilateral or a staged bilateral operation, although the bilateral pallidotomy was reported to have significant risks of severe side effects that included dysarthria, dysphonia, and cognitive decline;[143,164] consequently, the benefits of the second contralateral lesion remained questionable. Pallidotomy is not widely used at present, but unilateral pallidotomy has still been advocated by some as a more tolerable option compared to bilateral subthalamic stimulation, especially in older and more fragile patients, [144,165] although many controversies exist.[152] Additionally, at the beginning of the 1990s, alleviation of the motor symptoms of PD was shown in response to both chemical[81] (ibotenic acid) and thermal[166] lesioning of the STN, thereby verifying earlier results achieved by the creation of larger subthalamic area lesions.[147,153] Although STN lesions (STNL) are associated with dyskinetic complications and even hemiballismus, modern results suggest that unilateral STNL might at least be a relatively safe alternative[167], and that inclusion of the zona incerta (ZI) in the lesion could avoid dyskinesias.[168]

Despite some early, and mostly short-term, attempts at therapeutic stimulation,[169-171] the role of electrical stimulation in movement disorder surgery was viewed mainly as an intraoperative method to verify anatomical targets until the late 1980s.[172] Meanwhile, implantable stimulators had been developed, mainly for spinal cord stimulation for pain treatment.[173] This changed drastically after the reports of cessation of tremor during thalamotomy operations with test stimulation frequencies over 100 Hz by Benabid[174] and even earlier by Brice, who also reported 5 and 6 month results of two patients with implantable devices but external generators.[175] Benabid carried out experimental animal studies to show the effect of subthalamic deep brain stimulation (STN DBS) in a primate MPTP model of PD.[176] This led to clinical trials of STN DBS for PD[5] and the encouraging results awakened a new period of wider acceptance of surgical treatments for movement disorders. Since higher stimulation frequencies (>100 Hz) had a net effect similar to lesioning the targets, stimulation could now be used in a reversible manner to replace lesions.[177] The earlier success of pallidotomy led to trials of GPi DBS,[178-180] which proved to be an effective stimulation target for the reduction of L-DOPA-induced dyskinesias in PD,[181] in addition to being an effective target for dystonias.[182,183] The relative efficacy of STN DBS and GPi DBS has been studied in randomized controlled trials[184-190] and subsequent meta-analyses[191,192] showed that both targets lead to significant improvements, but only STN DBS has the possibility of reducing the need for dopaminergic medication. This implies that only STN DBS has a significant direct effect on PD symptoms, despite the earlier reports that pallidotomy reduced PD symptoms both in the pre-L-DOPA era[146] and in the DBS era.[193] Since its introduction, DBS has gained significant worldwide acceptance and

widespread use, especially for the treatment of advanced PD.[6]

2.2.1.1 A Linguistic notion

The proper use of the words "stereotaxis" and "stereotactic" has been historically controversial. Stereo and taxis are derived from Greek, while tactic is a Latin word for touch. Despite this combining of two different languages in a single word, the word stereotactic and its derivatives continue to be used. The use of the word stereotactic has been suggested to be reserved for human stereotaxis, while the use of stereotaxic is suggested for animal techniques.[194]

2.2.2 Clinical use of Deep Brain Stimulation in PD

Several guidelines or recommendations have been published on the use of DBS for PD.[195-196,197] Generally, the use of DBS has been confined to patients who have already developed LIDs or motor fluctuations.[195,197] The prerequisites for STN DBS are remaining effectiveness of L-DOPA [198] [195], no dementia, no significant psychiatric issues, and an ability to cope with the stimulator hardware and programming.[197] There have been suggestions and attempts to offer DBS also at earlier stages[199-201], and anecdotally, even prior to commencing L-DOPA. An earlier application of DBS has been suggested, especially in younger patients who are especially prone to develop severe LIDs.[199] The average time for a patient to proceed to DBS has remained high, at up to 10–15 years after diagnosis.[200] Although, in some cases, this long delay can reflect the slow disease progression by individual patients, there might still be a tendency to hold DBS as a last-resort therapy. On the contrary, it is commonly accepted that a follow-up period of 4–5 years is needed to ensure that PD diagnosis is confirmed and progressive supranuclear palsy and multisystem atrophy can be excluded.[12,195,202,203] Currently, other alternatives are available for patients with unsatisfactory L-DOPA medication effects: the most important of these are continuous intrajejunal L-DOPA-carbidopa gel infusion via an inserted percutaneous gastroenteric tube[204] and subcutaneous apomorphine.[197,205] Continuous L-DOPA infusion has been shown to reduce dyskinesias and motor fluctuations,[197,204] while apomorphine might be especially effective in treating non-motor symptoms during off periods.[197,205] These therapies are offered to partially overlapping populations, although patient characteristics and additional local practices can influence which therapy is favored over others.[206]

In current clinical practice, two main stimulation targets with their variations are used to achieve better control of the cardinal symptoms of PD: the subthalamic nucleus (STN) and globus pallidus interna (GPi).[192] Additionally, the Vim nucleus of the thalamus can be used to treat parkinsonian tremor, but it is more commonly used to treat essential tremor as it does not affect the other motor symptoms of PD.[207] However, some studies have reported that stimulation of the posterior subthalamic area between the classic STN target and Vim can provide better tremor control in some patients and has some effect on other motor symptoms.[208,209] A schematic presentation of the anatomical targets is shown in Figure 2. Other DBS targets have also been trialed in PD.[210] Both STN and GPi DBS are effective treatments, but only STN DBS produces direct improvement in PD symptoms and better reduction of dopaminergic medication, while GPi DBS leads to better improvement of LIDs and mood, with both targets providing similar overall benefit.[192] The side effect profiles seen with these targets differ: STN DBS is known to produce more cognitive and psychiatric side effects, in addition to the more commonly seen blurring of

speech.[192,211] Despite the suggestion that a choice between these targets could be made on individual basis, most centers seem to choose either STN or GPi as their main target for PD and offer this choice mostly to their patients.[212]

Patient selection plays a crucial role in the clinical success of DBS for PD.[197,198,202,213] The current recommendation is that expert multidisciplinary teams of neurologists and neurosurgeons should evaluate patients for DBS.[195,206,214] These teams can also include psychologists, nurses and physiotherapists. During the patient evaluation of a PD patient for DBS, the following issues have to be addressed:[197,198] The diagnosis of idiopathic PD is confirmed and patients with multisystem atrophy, corticobasal syndrome and progressive supranuclear palsy are excluded. The existence of major psychiatric comorbidities and cognitive decline are additional contraindications and have to be excluded. Neuropsychological tests or psychiatric consultations can be done routinely or on an as-needed basis as part of the evaluation. An MRI study of the brain is done to exclude significant brain atrophy suggestive of other neurodegenerative disorders and to exclude other significant brain diseases, such as tumors. A common prerequisite for PD DBS is a positive L-DOPA test, which assesses the remaining capacity of L-DOPA to reduce the motor symptoms of PD. Response to L-DOPA is the strongest predictor of a STN DBS therapeutic effect, in addition to age and disease duration.[196] The L-DOPA test is conducted by the neurologist usually in an inpatient setting, where all dopaminergic drugs are withheld overnight and, in the morning, an L-DOPA dose corresponding to 100%[195] to 150%[215] of the

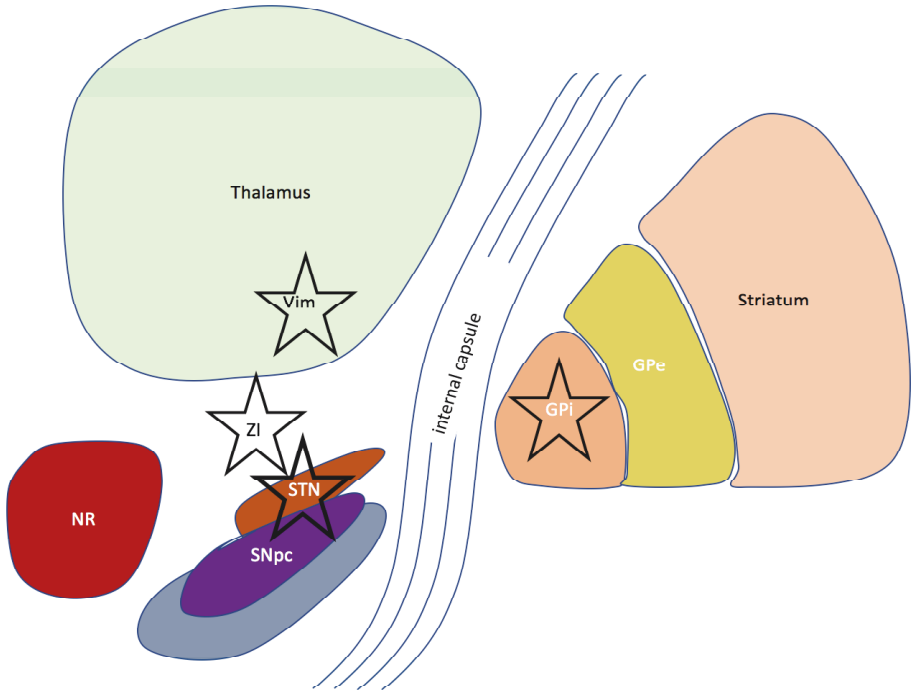

Figure 2 A schematic representation of the commonly used anatomical targets in Deep Brain Stimulation for Parkinson's Disease in the human brain. DBS targets are marked with a star. *GPe = globus pallidus externa, GPi = globus pallidus interna, NR = nucleus ruber, SNpc = substantia nigra pars compacta, STN = subthalamic nucleus.*

normal morning L-DOPA dose is administered. The motor symptoms are assessed by the neurologist on the UPDRS-III scale both before and after the L-DOPA dosing. A cut-off of 30% reduction in UPDRS-III is required for a positive L-DOPA response test.[202] After the neurologic screening, the functional neurosurgeon discusses the operation and related risks with the patient and the patient must understand and accept the 1–2% risk of symptomatic post-operative hematoma in the brain and the possible hardware and stimulation related complications.

The DBS surgery is mostly done with a frame-based stereotactic technique, although non-frame-based options have been described.[216] In the frame-based technique, the frame is fixated to the skull by four pins and the frame is localized by external fiducials on CT or MRI. If CT is used, then this is fused to a previously acquired MRI. The MRI sequences for DBS have been optimized to show the best possible anatomical resolution for the target (susceptibility weighted images for STN[217] and fast gray matter acquisition T1 inversion recovery[218]), normal white matter brain anatomy (T2-weighted), and vascular anatomy (T1-weighted gadolinium enhanced).[219,220] Classically, the implantation of electrodes is done under local anesthesia. The use of intraoperative microelectrode recording is advocated by some to verify the correct anatomic location of the electrode by recognizing the typical electric activity of the target nucleus.[221,222] Up to five separate microelectrodes in respective parallel tracts have been used. However, the use of a higher number of microelectrodes has been associated with a slightly increased of risk of post-operative intracerebral hematomas[223] and the omission of MER has not been associated with significantly worse results,[224] while MRI only series have resulted in better safety.[225] Intraoperative stimulation through permanent electrodes is often used to assess stimulation-induced side effects and possible therapeutic effects on the motor symptoms of PD[221] that might be difficult to assess during the operation. Sometimes, during intraoperative test stimulation or shortly after the STN DBS operation, stimulation-induced dyskinesias are encountered that are associated with a favorable outcome of STN DBS in the long-term.[226] If unsatisfactory side effects are encountered during testing, the electrode can be moved along the current tract or a new trajectory chosen for electrode placement.[221] Once the final location of the DBS electrodes is deemed satisfactory, the patient is put under general anesthesia for the implantation of the implantable pulse generator (IPG) that will provide continuous stimulation at the chosen parameters. The anatomical location of the electrodes is usually verified post-operatively by a CT scan.[227] However, the whole operation can be done under general anesthesia with a technique that relies completely on the imaging studies for electrode placement.[228,229] The accuracy of this technique can be improved by using intraoperative MRI to verify the correct anatomic placement of the electrodes.

The leads used for DBS classically have four electrodes for delivering the stimulation. The standard electrode used has 1.5 mm high electrodes with 0.5 or 1.5 mm distance between electrodes.[230] The presence of multiple stimulation electrodes permits adjustment of the exact anatomical target stimulated, as well as the choice of monopolar versus bi- or tripolar electrode configurations, which permit further tailoring of the produced stimulation fields and the anatomical effect of stimulation.[231] The common diameter of DBS leads is 1.27 mm, which is small enough to minimize the long-term microlesion effects of the electrode placement itself and a short-term microlesion effect predicts favorable outcome of STN DBS.[232,233] Electrodes permitting directional

steering of stimulation have been developed to achieve better therapeutic windows between symptom control and side effects.[230,234]

The stimulation parameters are usually optimized by a movement disorder neurologist and the PD drug treatment is adjusted[231,235] with the aim of decreasing the L-DOPA dose or, rarely, the complete cessation of dopaminergic drugs. The stimulation amplitude, frequency, pulse width, and active electrodes can be adjusted by programming the implanted pulse generator with an external programming device. The optimal stimulation parameters are usually found within 6 months after the implantation.[236] The implantable pulse generator requires a surgical change if the battery becomes depleted, which is seen, on average, in 3.5-5 years.[237] The battery life strongly depends on the individual stimulation parameters. As with any implantable devices, the DBS hardware can also become infected, which generally requires at least partial removal of the electrodes, although treatment with antibiotics only is sometimes successful.[238] Hardware-related problems have been reported as the most common type of complication.[239]

A recent meta-analysis found seven clinical trials comparing STN DBS versus best medical therapy (BMT).[240] However, none of these trials included sham operations and only one trial included delayed activation of stimulation at three months but without any blinding.[241] Additionally, the studies varied in design and follow-up times, which ranged from 3 months to five years (Table 2). The earliest randomized study with six-months of follow-up found reduced UPDRS-III scores at the medication OFF state, significantly improved quality of life, and reductions in troublesome dyskinesias.[185] However, the number of serious side effects was higher in the STN DBS treated group while treatment-related side effects were overall more common in the BMT group. Previous research reporting on cognitive and psychiatric side effects of STN DBS further verified that the improvements in motor symptoms and quality of life were not related to an overall deficit in cognitive functioning but to a selective decrease in frontal cognitive functions shown mainly by verbal fluency tests and variations of the Stroop test.[242] Additionally, anxiety was also improved. A later large multicenter open-label study with a well-controlled randomization protocol demonstrated similar improvements in QoL and motor function at 1 year, even when apomorphine treatment was readily available for both groups.[243] A direct comparison of STN DBS against apomorphine treatment with a very long 5-year follow-up, although without formal randomization and low number of patients, showed superior effects of STN DBS, especially for dyskinesias.[244] Additionally, the adherence to the assigned therapy was better in the STN DBS group. The effect of STN DBS in earlier stage PD was originally compared in a smaller trial[245] followed later by the EARLYSTIM trial with 2-year follow-up.[199] Although the smaller initial trial showed exceptionally good results on UPDRS-III in early PD, this effect was not fully replicated by the later, much larger trial.[199] However, because of the stringent use of blinded scoring of videos for UPDRS-III scores (apart from rigidity) under several treatment conditions, the EARLYSTIM trial provides perhaps the most robust evidence available for the efficacy of STN DBS over BMT on the motor symptoms of PD, since STN DBS is subject to the placebo effect.[246] Even though the EARLYSTIM trial showed a higher number of serious side effects in the STN DBS group, the number of serious stimulation- or medication-related side effects was lower for STN DBS than for BMT.

A recent meta-analysis comparing STN DBS with GPi DBS found several RCTs with some level of blinding. In these studies, the average LED reduction was 23.5% at 6 months and 32.8% at 12 months for STN DBS.[192] The overall treatment effects for STN DBS and GPi DBS were comparable, apart from a better LED reduction for STN DBS and lower depression scores for GPi DBS. Although the superiority of the targets remains controversial, both targets probably have their own merits[247,248] and could be chosen based on the personal characteristics of individual patients.[249] The therapeutic effects have been shown to last for up to 10 years and beyond,[250] but the progression of the disease leads to worsening of the motor symptoms, as shown by a gradual increase in UPDRS-III scores.[251,252]

The long-term stimulation-related side effects of STN DBS are mainly dysarthria, cognitive problems, and psychiatric symptoms.[12] Although stimulation-induced dyskinesia can be seen when the stimulation is turned on or the stimulation current is increased, stimulation-induced dyskinesias usually disappear. However, 2–4% of STN

Table 2 Improvement in motor score UPDRS-III after STN DBS vs. BMT in randomized trials in the MED OFF state. *STN DBS = deep brain stimulation of the subthalamic nucleus, BMT = best medical therapy, APO = apomorphine therapy, MED OFF = testing without dopaminergic medication*

Study	groups	3 months	6 months	12 months	18 months	24 months	5 years
Deuschl 2006[185] unblinded	STN DBS n=78		-41. 0%				
	BMT n=78		-14.1%				
Schübach 2007[245] unblinded	STN DBS n=10 MED OFF		-59%	-64%	-69%		
	BMT n=10 MED OFF		+6%	+ 8%	+ 29%		
Witt 2008[242] unblinded	STN DBS n=60		-44.1%				
	BMT n=63		-0.9%				
PD SURG trial 2010[243] unblinded	STN DBS/GPi DBS n=183			-35.7%			
	BMT n=183			-0.3%			
Okun 2012[241] unblinded	STN DBS immediate n=101	-39.3%					
	STN DBS delayed n=35	-12.1%					
EARLYSTIM 2013[199] blinded video	STN DBS n=124					-37.8%	
	BMT n=127					-3.9%	

DBS patients have been reported to suffer from long-term stimulation-induced dyskinesias.[253]

Considerable literature exists regarding the optimal anatomic target for STN DBS. Some candidates for the optimal target reside either on the dorsal border of STN[254] or outside the STN (e.g., the zona incerta).[255] A large single-center study that analyzed 262 STN DBS patients suggested better improvement when the electrodes were placed in the central associative area of the STN, as cognitive decline was found with more ventral and posterior electrodes.[256] Analysis of electrode repositioning in patients with poor symptom control suggested that sensorimotor STN might be the optimal target.[257] However, no consensus has yet been reached regarding the optimal target, although most specialists in the field place their preferred site for active stimulation inside the STN.[8]

The stimulation current also spreads to larger areas, so the stimulated area can include several anatomic structures, which affects the balance of therapeutic effects and unwanted side effects. Several anatomical structures have been related to both therapeutic and adverse effects of STN DBS. The posterior subthalamic area,[208] Vim,[258] and the dentato-rubro-thalamic tract[259] are known targets for parkinsonian and essential tremor located posterior to the STN. However, these targets are usually considered as separate targets from STN. The ansa lenticularis and lenticular fascicularis (field H2 of Forel) connect the GPi to the thalamus, as both run supero-medial to the STN in the subthalamic area and are separated from each other by the zona incerta and connected medially to form the thalamic fasciculus (field H1 of Forel).[260] The limbic STN, as the medial part of the STN, has been suggested to cause the psychiatric side effects of STN DBS.[261,262] However, the medial forebrain bundle running close to the medial STN has also been suggested to convey some of the psychiatric side effects of STN DBS.[263] Both the pars reticulata and pars compacta parts of the substantia nigra are located inferomedially to the STN, and the simultaneous use of stimulation of SNpr during STN DBS has been studied to alleviate refractory gait disturbances.[264] The spread of current to the corticospinal and corticobulbar tract axons located in the cerebral peduncle can cause contralateral muscle contractions.[265,266]

The medial lemniscus is located in the posterior subthalamic area, and it conveys sensory axons from the spinal cord to the sensory thalamus. The spread of current to the medial lemniscus causes paresthesias.[267] The nucleus ruber can be inadvertently stimulated by medial contacts to produce gait and postural ataxias.[268] Projections from prefrontal areas (i.e., the frontal eye fields) to the oculomotor nuclei run partly in the subthalamic area, and their stimulation can cause conjugate eye deviation that is commonly controversive and habituates easily.[268] This contrasts with third nerve fiber stimulation from a more medial stimulation, which is ipsiversive, manifested only in the ipsilateral eye, and does not habituate. Blurring of speech, or dysarthria, can occur with electrodes located within or outside the STN.[269] During intraoperative test stimulation, these side effects can be used to confer the anatomic location of the electrode and help with decision making related to the final electrode positioning and possible electrode repositioning.

2.2.3 Experimental Deep Brain Stimulation

The era of modern clinical DBS was initiated partly by animal research in non-human primates[270] to confirm the beneficial effect of STNL and STN HFS. Later studies focused more on the neuro-electrophysiological and biochemical mechanisms of stimulation and possible neuroprotective effects. In non-human primates and other large animals, the use of implantable electrodes and pulse generators similar to those used in human clinical DBS is possible.[271] By contrast, the use of rodents as experimental animals requires miniaturized electrodes and most often the use of external impulse generator that is tethered to the electrode.[272] Primate studies are expensive and require extensive preparations to ensure animal welfare and ethics, whereas rodent studies are less expensive and the animal welfare considerations are easier to meet.[273]

Experimental DBS is often called high frequency stimulation (HFS) to delineate the therapeutic effects seen with higher frequencies over about 60 Hz from the opposite effects seen with low frequency stimulation.[272] Experimental rodent studies have mostly been conducted with external pulse generators; however, implantable pulse generators have been used by some groups. [274,275] The use of an external pulse generator requires the use of a tethering mechanism and solving the problem of tangling of the tethering leads that connect the pulse generator to the electrodes, which are fixed to the skull of the experimental animal. In some studies, this issue has been solved by conducting the whole experimental stimulation under general anesthesia[276,277] and in others by using an electrical swivel that permits the rotation of the leads in a freely moving animal. [278-280] However, the use of an external pulse generator restricts the duration of the stimulation experiment.

Lesioning of the target nucleus can also be used as an experimental model of long-term DBS as it shares some of the effects seen with stimulation, even though lesioning and stimulation also have distinct mechanisms.[177,281]

2.3 Neuroprotective Deep Brain Stimulation

The decrease in dopaminergic tone in the parkinsonian striatum leads to disinhibition of the STN. This was hypothesized to lead to increased excitotoxicity in the SNpc, which, together with neuroprotection seen with NMDA antagonists, provided the initial rationale for trialing STNL for neuroprotection in animal models of PD.[282-284] The first published study on the subject reported a neuroprotective effect seen only in preventing SNpc dopaminergic cell loss (-8% vs. -41% in the sham group), but no reduction was noted in the loss of striatal tyrosine hydroxylase (TH)-positive neurites. A later study found that STNL performed 7 days before striatal 6-OHDA (4 × 7 µg) injections could rescue dopaminergic cells in the SNpc (+23% compared to sham STNL) and improve contralateral front limb use, but it did not decrease amphetamine- and apomorphine-induced rotations.[285] However, STNL had a neuroprotective effect only when performed before the 6-OHDA lesion instead of two weeks after the 6-OHDA lesion. Another study that analyzed the time scale of neuroprotection with repeated behavioral tests found the greatest neuroprotection at 2 weeks, after which it diminished.[286] Similar results have been found in MPTP primate trials, where chronically stimulated or STNL-treated monkeys showed 20–24% more TH positive cells in the SNpc when compared to sham treated monkeys.[271] A later report indicated an increase in TH-positive cells in the periaqueductal gray.[287] In animal studies, the ability of STNL to protect SNpc dopaminergic neurons in relatively limited

striatal 6-OHDA lesions was related to the timing of STNL in relation to the 6-OHDA injections, which might reflect a relationship between the neuroprotective effect of STNL and the depth of the dopaminergic degeneration or that STNL is only able to prevent or postpone the dopaminergic lesion rather than restore it.

The neuroprotective results of STNL were later replicated in studies using electrical stimulation of the STN. Chronic STN HFS for 2 weeks started immediately after striatal 6-OHDA lesion was found neuroprotective increasing the SNpc TH positive cell count from 43.5% to 84.8%.[288] Intermittent bilateral STN HFS for 3 months, started 1 week after bilateral low dose 6-OHDA, reduced dopaminergic cell death by 28%.[289] Continuous STN HFS initiated 2 weeks after lesioning in a very-low-dose 6-OHDA (2 × 1 µl) striatal rat model was neuroprotective, increasing the number of TH positive cells in SNpc by about 40% when compared to sham stimulation.[290] However, no changes were noted in numbers of striatal TH positive neurites or in striatal dopamine levels. In a more pathologically realistic alpha-synuclein model of PD, STN DBS started three weeks after AAV1/2-A53T alpha-synuclein protected dopaminergic neurons in the SNpc.[291] The neuroprotective effect of STN HFS has been recently shown to be mediated by a growth factor receptor trkB.[292]

However, clinical studies have failed thus far to reproduce these neuroprotective effects.[200,293 294] On repeated ^{18}F-fluorodopa PET, patients treated with STN DBS continued to show decreasing dopaminergic function over 16 months.[295] A retrospective case control study with 81 treated patients concluded that STN DBS does not slow the progression of symptoms and has no statistically significant effect on mortality over an average follow-up period of 7.5 years.[252] This contradicted an earlier study where stabilization of PD symptoms with stimulation OFF was found after STN DBS when compared to baseline in a smaller group of 21 patients with a follow up for 1–5 years.[296] However, in that study, only a 30-minute washout period was used after turning STN DBS off before conducting clinical tests for stimulation OFF status.

In clinical practice, the delay from PD diagnosis to DBS can be up to 14–15 years, on average.[200] As described previously, post-mortem analysis has shown that dopaminergic degeneration is almost complete at about 5 years after diagnosis.[11] This might explain why no neuroprotective or neurorestorative effects have been seen for STN DBS in clinical trials. The EARLYSTIM trial[199] led to FDA approval of earlier use of STN DBS in patients with at least 4 years of disease duration[297] and this can lead to better opportunities to uncover the possible clinical neuroprotective effects of STN DBS.

2.3.1 Mechanisms of the effect of subthalamic deep brain stimulation

Originally, the clinical use of DBS was initiated when high frequency stimulation was noted to mimic reversibly the effects of a thalamic lesion.[239] This finding led to a heuristic mechanism that DBS was a simple lesion analogue, although more complex mechanisms were soon discovered. [298] The question of the exact mechanisms of the effect of DBS is a complex issue that can be approached from various viewpoints, such as electrophysiological local effects on neuronal components around the electrode,[299] electrophysiological effects distant to the target nucleus,[300] effects on neurotransmitters,[301] effects on intracellular signaling, effects on gene expression[279] and protein synthesis, network level effects,[302] and acute vs. chronic effects.[303] Some of the research questions are impossible to study in

human PD patients, so these have to be addressed in animal experiments.

DBS conducted with the clinically most commonly used frequencies of over 100 Hz generally produces very similar effects to similarly located lesions; however, the exact nature of the impact of DBS on the different neural components has been the subject of considerable discussion and remains incompletely understood.[304,305] Furthermore, different neural structures (neuron somas and axons) probably respond differently to stimulation.[299]

The overall electrophysiological effect of STN DBS has been proposed to be a balancing of an abnormally functioning neural network by jamming the abnormal bursting activity in the STN. [306] The effects of STN DBS on other components of the motor CTSC have been studied extensively: STN DBS has been shown to disrupt the electrophysiology of the GPi[307] and thalamus [308] and the excitability of the motor cortex,[309,310] to decrease the pathological beta-band synchronization of the motor CSTC,[311 312] and to reduce the GABA content in the ventral anterior nucleus of thalamus in a manner similar to L-DOPA.[313]

In 1986, STN HFS was reported to affect dopamine release, but the results were controversial: a significant increase was noted in DA in the SNpc but the striatal DA levels decreased.[314] Later studies reported increases in the levels of the striatal dopamine metabolites, DOPAC and HVA, without any increase in the striatal DA in both native and 6-OHDA-lesioned rats.[315,316] The inhibition of dopamine uptake by nomifensine revealed an increase in striatal DA during STN HFS in striatum 6-OHDA-lesioned rats.[317] Interestingly, small changes in the exact position of the electrode within DBS has been reported to either increase or reduce dopamine release in the striatum in non-human primates.[318] However, despite some encouraging results,[319] STN DBS does not seem to increase striatal DA in the human striatum.[320]

The levels of glutamate and GABA are reported to increase in the SNpr but only the levels of glutamate increase in the GPe.[321] This increase of glutamate in SNpr has been related to stimulation-induced dyskinesias,[322] and a broad-spectrum glutamate antagonist was able to prevent stimulation-induced dyskinesias. Interestingly, in the same study, the GABA levels in the SNpr were reported to increase only when stimulating with a low amplitude that did not induce dyskinesias. However, the increase in SNpr glutamate and GABA persisted after stopping the STN HFS, underscoring that additional mechanisms are involved in the behavioral effects of STN HFS.

STN HFS-induced rotations are reduced by intrastriatal injections of D2 antagonists but not by D1 antagonists in rats.[323] In a 6-OHDA hemiparkinsonian rat model, STNL decreases the phosphorylation of thr34 in DARPP-32 that is related to L-DOPA-induced dyskinesias caused by intermittent L-DOPA administration, although STN had only a minor effect on dyskinesias.[86] In animal models, STN DBS induces changes in striatal gene expression[279] and, interestingly, increases the striatal BDNF levels in native but not in 6-OHDA lesioned rats after two weeks of continuous stimulation.[324]

Overall, STN DBS appears to have a widespread effect on all components of the motor CTSC that can be seen in various facets of neural functioning, ranging from electrical activity to gene expression.

2.4 Neurotrophic growth factors

Neurotrophic factors (NTFs) are secreted neuropeptides or proteins that are found in the CNS and the PNS, where they regulate the growth, survival, and function of

neurons.[325-327] Nerve growth factor (NGF), discovered in 1956, was the first NTF to be found. [327] NGF binds to the tropomyosin kinase receptor A trkA, a transmembrane protein that mediates the effects of NGF on intracellular trophic signaling pathways.[327]

The glial derived neurotrophic growth factor (GDNF) family comprises the proteins GDNF, neurturin, artemin, and persephin.[325] GDNF exerts its effects by binding to a complex comprising the GDNF family receptor-α and receptor tyrosine kinase, both of which are expressed by dopaminergic neurons. [328] GDNF and neurturin are neuroprotective *in vitro* and *in vivo* in Parkinson's disease models. [14] GDNF reduces ROS formation in 6-OHDA PD models.[329] Neurturin binds to the naturally occurring heparan sulfate proteoglycan in the brain.[330] Additionally, GDNF, GDNF family receptor-α and receptor tyrosine kinase have wide effects throughout virtually all cell types and organs in the body.[325]

A novel neurotrophic factor, conserved dopaminergic neurotrophic factor (CDNF, also originally termed cerebral dopaminergic neurotrophic factor and used synonymously), and later, a related mesencephalic astrocyte derived neurotrophic factor (MANF) were described with qualities differing from the GDNF family.[326] The CDNF/MANF family NTFs have no known receptor, but they probably exert their effects in the cytosol or the endoplasmic reticulum of neurons. Unlike GDNF and NRTN, neither CDNF nor MANF binds to heparin sulfate proteoglycans in the brain and this feature has been related to their better diffusion in the brain parenchyma.[331] CDNF and MANF are both neuroprotective and neurorestorative in *in vitro* and *in vivo* models of PD. Recently, CDNF has shown a neuroprotective interaction with GDNF[332] and with MANF[333].

Preclinical studies have examined the effects of administration of NTFs as single injections or continuous infusions of the proteins themselves or viral constructs.[14] Neurturin variants have also been studied, in part, to overcome the problem of diffusion in the brain.[334]

2.4.1 Clinical trials of neurotrophic growth factors

An initial open label clinical phase I trial[335] and a phase II trial[336] of continuous infusions of GDNF protein to the putamen showed a positive effect on PD symptoms. However, this was not replicated in a subsequent RCT.[337] Some patients in these trials developed anti-GDNF antibodies and the primate models developed cerebellar atrophy soon after the GDNF therapy was stopped.[338] Several possible reasons for the failure of GDNF therapy were suggested.[328,339] A RCT of an AAV2-NRTN construct administered bilaterally to both the SNpc and putamen[340] revealed no difference in the motor symptoms of PD (UPDRS-III). However, a statistically significant difference was noted in two of the secondary end point complications of therapy (UPDRS-IV) and in the time at home without troublesome dyskinesia at 15 months after the injections when compared to sham surgery. Overall, a recent meta-analysis found no proof of an effect of NTFs on PD.[341]

Interestingly, a statistically significant improvement was noted in the motor symptoms compared to baseline in both groups, and the patients who received neurturin-AAV therapy less than 5 years after PD diagnosis had a statistically significant benefit according to a post-hoc analysis.[13] This effect was found also in a reanalysis of a previous Phase II study. However, post-mortem analysis revealed increases in TH level around the injection sites, suggesting that limited diffusion of NTF or AAV-NTF

constructs could explain, at least in part, the negative results of the clinical trials.[13,342] This limitation could possibly be overcome by using NTFs with better capability of diffusing in the brain, such as CDNF, or using smaller targets in the dopaminergic system, such as the SNpc.

3 AIMS OF THE STUDY

STN DBS has been shown to be effective as a treatment in clinical and experimental PD, but the optimal clinical target, behavioral effects in animal models, and neuroprotective potential remain incompletely described. Various NTFs have shown effectiveness in partial dopaminergic degeneration models of PD, but the effects in late stage models have been mostly unexplored. Furthermore, in the late stages of PD where dopaminergic cell death is almost complete, the possibilities of neurorestoration are limited. The capabilities of NTFs in enhancing the effect of STN DBS have not been previously studied.

The specific aims of the study were:

I) To better describe the behavioral effects of STN DBS in a rat 6-OHDA model of late stage PD.
II) To further analyze the therapeutic effect of clinical STN DBS and compare different methods of electrode location.
III) To study the effects of the combination of CDNF and STN DBS in an animal model of late stage PD.

4 MATERIALS AND METHODS

4.1 Animals

Wistar rats acquired from Harlan/Envigo (The Netherlands) were used in all experiments. The animals were housed in cages in groups of four, unless otherwise mentioned, in a 12:12 hour light cycle. All surgeries and behavioral experiments were done during the light period. Food (Teklad, Harlan Envigo, Indianapolis, IN, USA) and water was available ad libitum during all experiments. After electrode implantations, the animals were individually caged for the rest of the experiment to prevent chewing of electrode contacts by cage mates.

4.1.1. Rodent stereotaxy

All stereotactic surgery was done under general isoflurane anesthesia using a rodent stereotaxic frame (Stoelting, Wood Dale, IL, USA). All coordinates were measured from the bregma and based on previous studies and the Watson & brain atlas for rats.[343] Injections were made using an automatized injector (Quintessential Stereotaxic Injector; Stoelting, Wood Dale, IL, USA or Micro4 and UMP3; WPI, Sarasota, FL, USA) and 26g syringes (Hamilton, Bonaduz, Switzerland).

4.2 Experimental toxins, neurotrophic factors, and drugs

The following toxins and drugs were used in the animal experiments: 6-hydroxydopamine, amphetamine, apomorphine, buprenorphine, carprofen, chloral hydrate, ibotenic acid, isoflurane, lidocaine, pentobarbital, and tramadol.

4.2.1 6-OHDA lesion

6-OHDA was diluted to 0.02% in an ascorbic acid PBS solution, and protected from light and kept on ice until infusion. Stereotactic infusions were made with a Hamilton 26g needle to inject 8–10 µg of 6-OHDA (2µg/µl) at a rate of 1µl/min, and the infusion needle was kept in the target area for 2 min after infusion was stopped to prevent backflow. Injections were done into the anterior medial forebrain bundle (mfb) (A/P -2.0; M/L +2.0; D/V -8.3) or into the posterior mfb (A/P -4.4; M/L +1.2; D/V -8.3). All STN HFS experiments were done with the anterior mfb injection and NTF-only experiment was done with the posterior target.

4.2.2 Ibotenic acid lesioning of the STN

Stereotactic injections of 10 µg ibotenic acid (10µg/µl; #329130010, Acros Organics Belgium) diluted in PBS were made to the STN (A/P -3,6; M/L +2.3; D/V -8.0) at a rate of 1µl/min. The infusion needle was kept in the target for 2 min after the infusion was stopped. The excessive rotational behavior after ibotenic acid infusion was treated with i.p. injection of 4% chloral hydrate (100–150 mg/kg; Sigma-Aldrich #C8383, St. Louis, MO, USA).

4.2.3 CDNF and GDNF delivery

Recombinant proteins CDNF and GDNF dissolved in PBS were infused just above the SNpc, ipsilateral to the 6-OHDA lesion (A/P -5.4; M/L 2.0; D/V -8.3) at a rate of 0.5 µl/min, and the infusion needle was kept in place for additional 4 minutes after infusion to prevent backflow.

4.2.4 Amphetamine and apomorphine

Amphetamine and apomorphine were dissolved in PBS and injected intraperitoneally to study drug-induced rotations.

4.2.5 Animal anesthetics and analgesics

For stereotaxic surgeries, the rats were placed under isoflurane general anesthesia, buprenorphine or tramadol analgesia, and local anesthesia with lidocaine. Postoperative analgesia consisted of subcutaneous carprofen, buprenorphine, or tramadol. Intraperitoneal pentobarbital overdoses were used solely for euthanasia. Choral hydrate was used for sedation after STNL operations to reduce excessive rotational behavior.

4.3 Experimental deep brain stimulation

4.3.1 Electrodes

Implantable bipolar electrodes (MS308/2, Plastics1, Roanoke, VA, USA) made for animal use were used for all experimental stimulations. The electrodes were implanted into the STN (A/P -3.6; M/L 2.3; D/V -8.3; in relation to bregma) and fixated to the skull with dental cement and screws.

4.3.2 Stimulation parameters

All experimental stimulations were conducted at a frequency of 130 Hz and pulse width of 60 µs. The stimulation amplitude was chosen individually for each rat based on stimulation-induced dyskinesias. Stimulation-induced dyskinesias were tested by stepwise increases of the stimulation amplitude from 0 to 400 µA. Two stimulation amplitudes were chosen: a low amplitude corresponding to minimal dyskinetic response and a high amplitude corresponding to the amplitude just below the appearance of notable front limb dyskinesia.

4.4 Western blotting

Animals were euthanized by decapitation for western blotting analysis. The heads were cooled immediately in liquid nitrogen and the brains were dissected rapidly, flash frozen in precooled isopentane (-40°C), and put on dry ice before storage at -80°C. Samples for western blotting were harvested from frozen brains in cryostat at -14°C by taking 3 mm punches to approximately a 2mm depth. The samples were kept on dry-ice and stored at -80°C until they were lysed with mechanical shearing and boiling in 1% SDS PBS. The protein content was measured with a DC Assay (Bio-Rad, Hercules, CA, USA) and a UV-2401PC spectrophotometer (Shimadzu, Kioto, Japan) and UV Probe 2.31 software (Shimadzu). A 20 µg sample of protein was loaded onto 10% TG gels (Bio-Rad) and blotted onto PVDF (polyvinylidene difluoride) membranes (Bio-Rad) with a Turboblotter (Bio-Rad). Signals were detected with ECL+ chemifluorescence and a Typhoon 9000 (GE Lifesciences, Chicago, IL, USA). The signal intensity was quantified with ImageJ software. [344].

4.5 Histology

Electrode tip locations were confirmed by histologic sections of the subthalamic area. Samples for histologic staining were acquired from the same brains that were used for western blotting. Sections were cut 40 µm thick in cryostat at -14°C, mounted on coated glass slides, air-dried overnight, and stored at -20°C until processed. Before staining, the sections were thawed and air-dried for 1 hour. After post-fixation with 4% PFA (paraformaldehyde) in PBS for 20 min, and washing with PBS and milliQ water, the sections were stained with 0.2% cresyl violet for 20 minutes, differentiated and dehydrated in an ethanol series, and fixed with xylene. Sections were mounted with DP mounting media (Sigma-Aldrich , St. Louis, MO, USA) and covered with a coverslip.

Electrode tip location was analyzed under 5 light microscopy, and the location of the

electrode was judged by anatomical tip location according to Paxinos and Watson.[343]

4.6 Immunohistochemistry

The animals were euthanized with an overdose of pentobarbital (90 mg/kg, i.p., Mebunat®, Orion Oyj, Espoo, Finland) and perfused transcardially with 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde overnight, and stored in 20% sucrose at 4°C. The striatum and substantia nigra regions of the frozen brains were cut into 40 µm thick coronal sections in series of six with a gliding microtome (Leica Biosystems, Newcastle, UK).

Free-floating sections were stained with mouse anti-TH antibody (1:2000, MAB318, Millipore, Billerica, MA, USA) or anti-DAT antibody (1:2000, MAB369; Millipore). The endogenous peroxidase activity of the free-floating sections was quenched for 30 min in 0.3% hydrogen peroxide solution for TH staining. For the anti-DAT staining, the sections were incubated in 10 mM citrate buffer (pH 6.0) at 80°C for 30 min. The sections were incubated in 4% BSA and 0.1% Triton- -100 to block unspecific binding. Primary antibody incubation was overnight. Following the incubation in the biotinylated secondary antibodies (1:200, anti-mouse or anti-rat, Vector Labs, Burlingame, CA, USA), the signal was enhanced using the avidin-biotin-enzyme complex (ABC-kit, Vector Labs) and visualized with 3',3'-diaminobenzidine. The sections were mounted on coated glass slides and covered with coverslips.

Images of TH- or DAT-stained sections were acquired with digital camera (Nikon Corp., Tokyo, Japan) attached to a stereomicroscope (Nikon). For experiment 3 in publication II, the images of the stained sections were acquired with a 3DHistech scanner (3DHistech, Budapest, Hungary) located in the Institute of Biotechnology (http://www.biocenter.helsinki.fi/bi/histoscanner/index.html). The optical density of the TH-positive fibers in the striatum was determined from six sections from each rat using the ImagePro software (Media Cybernetics, Inc., Rockville, MD, USA). The results are given as a percentage of the intact side, which is defined as 100%. For analysis of optical density, the corpus callosum was defined as the zero level.

The number of TH-positive cells in the SNpc was counted using the StereoInvestigator software (MicroBrightfield, Williston, VT, USA). TH-positive cells in the SNpc were counted bilaterally from six sections. The data are presented as a percentage of the intact side.

4.7 High-pressure liquid chromatography (HPLC)

The HPLC analysis was done as previously described, with the following protocol.[345] The rat striata samples were stored at -80°C and later homogenized with an ultrasonic homogenizer (Rinco Ultrasonic AG, Romanshorn, Switzerland) in a homogenization solution consisting of six parts 0.2M $HClO_4$ and one part antioxidant solution (1.0 mM oxalic acid, 0.1 M acetic acid, 3.0 mM L-cysteine). After centrifugation at 20,800 g for 35 min at 4 °C, the supernatant which was transferred to 0.5 ml Vivaspin filter concentrators (10,000MWCO PES; Sartorius, Stonehouse, UK) and centrifuged at 8600 g at 4 °C for 35 min. High-pressure liquid chromatography (HPLC) using electrochemical detection was used to analyze monoamines from the filtrates. Analyses included the concentrations of dopamine (DA) and its main metabolites (3,4-dihydroxyphenylacetic acid [DOPAC] and homovanillic acid [HVA]) and serotonin (5-HT) and its main metabolite (5-hydroxyindoleacetic acid [5-HIAA]). The

column was a Phenomenex Kinetex 2.6 µm, 4.6 × 100 mm C-18 column (Phenomenex, Torrance, CA, USA), held at a temperature of 45 °C using a column heater (Croco-Cil, Bordeaux, France). The mobile phase consisted of 0.1 M NaH_2PO_4 buffer, 120 mg/l octane sulfonic acid, methanol (5%), and 450 mg/l EDTA, with the pH adjusted to pH 3 with H_3PO_4. The flow rate of 1 ml/min was maintained with a pump (ESA Model 582 Solvent Delivery Module; ESA, Chelmsford, MA, USA) and an autoinjector (SIL-20AC, Shimadzu, Kyoto, Japan) injected 100 µl of the filtrate into chromatographic system. An electrode array detector with 12 channels (CoulArray, ESA, Chelmsford, MA, USA) was used for monoamine and metabolite detection. DA was detected in channel 2 (applied potential 80 mV), DOPAC in channel 3 (applied potential 120 mV), 5-HT and 5-HIAA in channel 4 (applied potential 160 mV), and HVA in channel 8 (applied potential 320 mV). CoulArray for Windows software (ESA, Chelmsford, MA, USA) was used for chromatogram processing and calculations of monoamine concentrations. The concentrations of analytes were expressed as ng/g wet tissue.

4.8 Behavioral assays

4.8.1 Pharmacologically induced rotations

The level of the dopaminergic lesion was assessed by inducing a rotation either with amphetamine (2.5 mg/kg s.c.; Sigma-Aldrich, Saint Louis, MO, USA) or with apomorphine (0.1 mg/kg; Sigma Aldrich, Saint Louis, MO, USA). The rotations were measured with an automated rotometer (MedAssociates, Inc. St. Albans, VT, USA) to which the rats were connected by a vest. The baseline rotations were measured for 30 minutes before injections of amphetamine or apomorphine. Pharmacologically induced rotations were measured for 60 minutes.

4.8.2 Stimulation-induced dyskinesia

Stimulation-induced dyskinesias were assessed both live during step-wise increasing currents (0-400 µA) and later from videos. Dyskinesias were assessed separately for orofacial, axial, front limb, and locomotive subtypes, as previously described. [88] The scale (0–4) was adapted and modified from Cenci. The scale used was: 0= no dyskinesia; 1=transient (<2s) or unclear; 2=infrequent and mild or decays strongly (<10s); 3=marked, long lasting (>10s), frequent (>50%) and 4=extreme, minimal decay (>60 s), almost all of the time. The stimulation amplitude was increased step-wise at intervals of 15 sec (0, 25 ,50 ,75 ,100, 150, 200, 250 ,300, 350, and 400 µA) or until the dyskinesias reached a maximal level on the scale. The total number of dyskinesias were presented as a sum of all dyskinesia ratings across all stimulation amplitudes. In the video analysis, orofacial dyskinesias could not be reliably assessed. We used Cronbach's alpha to test for a scale reliability and accordingly used a sum of axial, front-limb, and locomotive dyskinesias to represent the final measure of stimulation-induced dyskinesias.

4.8.3 Cylinder test

The front-limb use asymmetry was studied by counting contacts made with the cylinder wall with the front limbs. Rats were placed in transparent cylinders 20 cm in diameter. The rats were able to move freely in the cylinders. Video was recorded from underneath for 10 minutes for each cylinder test session. The response of front limb use asymmetry to STN HFS was tested on consecutive days for baseline condition (no stimulation, stimulation lead connected to the electrode), low stimulation amplitude, and high amplitude, chosen individually based on stimulation-induced dyskinesias.

The cylinder videos were scored so that the researcher was blinded to the dyskinesia ratings and whether rat had received CDNF or IBOT or vehicle in experiments where the interaction between STN HFS and CDNF treatment was studied. The contacts made with the wall during rearing movements were counted separately for the first contact with the wall during individual rearings and for all contacts the rat made with the forepaws on the wall during rearing movements. The touches with the wall were counted as double only if the contact was made during consecutive frames in a 29 fps video. All further statistics were conducted based on front limb use asymmetry, where each double touch was allocated evenly to the left/right front limb (100*(right+double)/(right+2*double+left)).

4.9 Patient recruitment and assessment of motor symptoms

The Helsinki University Hospital Neurosurgery department operative records were searched for STN DBS operations for PD between 2007 and 2014. In total, 103 patients were found and their patient files were reviewed and imaging studies analyzed. In 87 cases, complete data were available and the imaging quality was sufficient to analyze the location of the active electrode. The patients were screened at the Helsinki University Hospital Neurology department. The inclusion criteria were: idiopathic PD and disease of 5 years and severe dyskinesia, daily ON/OFF fluctuations or drug resistant rest tremor related to L-DOPA treatment and leading to suboptimal treatment effect. Improvement of over 30% in the UPDRS part III score (UPDRS-III) after a levodopa challenge test was also required. Contraindications were dementia, severe psychiatric comorbidity (psychosis or severe depression), clinical or radiological suspicion of atypical parkinsonism, and a significant brain atrophy or vascular changes in brain MRI.

The primary motor outcome of STN DBS was assessed using UPDRS-III. The preoperative UPDRS-III in the medication OFF state was chosen for evaluating the baseline severity of Parkinson's disease. The motor outcome was evaluated at six months in the medication OFF and DBS ON state. The secondary treatment outcomes were changes in the Hoehn and Yahr (H&Y)stage derived from patient files and dopaminergic medication was calculated as levodopa equivalent dose (LED) [346]. The patient files were searched for stimulation settings (voltage, pulse width, polarity and frequencies) at six months and these were used for further analysis. Complications, including intracerebral hemorrhage (ICH), infection, and dysarthria, were meticulously searched for from patient files and imaging data. Clinical episodes requiring treatment with antibiotics and/or revision of the wound or the DBS hardware were defined as infections.

4.10 Clinical STN DBS surgery and programming

The DBS operation and perioperative imaging were conducted according to clinical practice and the choice of the operating surgeon. Preoperative brain MRI was used in most cases for planning after merging to a CT scan acquired with Leksell stereotactic frame on the morning of the DBS operation. The targeting was commonly done by two surgeons independently and the final coordinates were decided by comparing the two coordinate sets. The target coordinates were calculated in relation to the midcommissural point (MCP) in the anterior commissure–posterior commissure (AC–PC) coordinates and by a method based on direct MRI visualization of the subthalamic nucleus (STN) [217] and/or the nucleus ruber (NR), [347] where image quality of the brain MRI

permitted. The implantation and testing of the DBS electrodes was done under local anesthesia and light sedation. After electrode implantation, -ray fluoroscopy in the AP and lateral directions was used intraoperatively to verify correct electrode placement in the stereotactical space. After fluoroscopy, all four contacts were tested for side effects and clinical benefit while the patient was awake. The patient was put under general anesthesia for implantation of the impulse generator (IPG).

4.11 Electrode location analysis

Electrode location was analyzed from routinely acquired MRI and CT scans and no additional imaging was done for this study. Post-operative CT scans (performed on the first or second postoperative day) were reviewed for complications and to determine the amount of intracranial air using the Agfa Healthcare N.V. Impax software (version 6.5.5.1608, Belgium). The midline shift was measured from preoperative and postoperative CT images and the thickness of subdural air collection was measured from the postoperative CT image. Post-operative thin slice CT images were fused to preoperative MRI scans and re-aligned to the AC–PC orientation for electrode location analysis with the Brainlab iPlan (version 3.0.5, Germany) stereotactic software. Where pre-operative DBS images were of suboptimal quality, we used the previous 3 Tesla MRI scans obtained during screening. The anterior and posterior commissure were identified preferentially from 1.5 Tesla 3D T2 image to define MCP. Direct visualization of STN and electrode location in relation to NR was done preferentially from 1.5 T susceptibility weighed images (SWI) [220]. The active electrode (defined as the negative electrode) at six months was used for electrode location analysis. In cases with two active negative electrodes, the mid-point between these electrodes was defined as the electrode location. The use of bipolar or monopolar stimulation was recorded.

The location of the active electrode was determined in three coordinate systems: direct visual analysis of the MRI scans, which was correlated by the researcher to the Mai atlas [348] in coronal orientation, the location in relation to the NR (distance from anterior, lateral, and superior borders), and location in relation to MCP. The electrode location was recorded as -, Y-, and Z-coordinates representing the mediolateral, antero-posterior, and dorso-ventral directions. The median coordinates of the electrodes were compared between patients with less than 30% and those with 30% or more reduction in LED and UPRDS-III scores between the baseline and at 6 months.

4.12 Statistics

All statistical analyses were done using SPSS 24.0 software (IBM, Armonk NY, USA). Normality of variables was tested with the Shapiro-Wilks test and equality of variance with Levene's test; parametric and nonparametric statistical tests were used where appropriate. An independent sample t-test or the Mann-Whittney U test was used to compare variables with two independent groups and a paired sample t-test or Wilcoxon signed rank test was used to compare repeated measurements. In cases of multiple groups, ANOVA was used, followed by Tukey's post hoc test to allow for comparison between all study groups or Dunnett's test for comparison of treatment groups against a control group. Friedmans's test was used to compare multiple groups in cases of nonparametric variables. Correlations were calculated using Pearson or Spearman correlation. Lin's concordance co-efficient correlation [349] was used to analyze the agreement between two observers for dyskinesia scoring of stimulation-induced dyskinesias in rat.

4.13 Ethics

The animal experiment designs and procedures in publications I and II were approved by the Committee for Animal Experiments of the University of Helsinki, by the chief veterinarian of the County Administrative Board for 2008–2010, and by the National Animal Experiment Board for 2011–2015. Animal experiments were conducted according to EU regulations (EU Directive 2010/63/EU) and Finnish legislation (Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes [497/2013] and the Government Decree on the Protection of Animals Used for Scientific or Educational Purposes). The clinical study design in publication III was approved by the HUH Medical Ethics Committee.

5 RESULTS

5.1 Stimulation-induced dyskinesias

Implanting STN HFS electrodes at six weeks after 6-OHDA lesion (Figure 3A) and increasing stimulation amplitudes produced gradually increasing stimulation-induced dyskinesias in all rats (difference from baseline after 100 µA $p < 0.001$). On average, the orofacial dyskinesias appeared first, followed by axial and front limb dyskinesias, and then locomotive dyskinesias. Individual variance was noted in the severity of dyskinesias and in the order of appearance of the dyskinesia subtypes (Figure 3B).

The dyskinesia subscale ratings (orofacial, axial, front limb, and locomotive) showed a high Cronbach's alpha with both live and video-based scoring (0.942 and 0.913). This was slightly improved by removing the orofacial dyskinesias (0.953 and 0.913, Figure 3C). As the orofacial dyskinesias also proved difficult to score from the video, we used the sum of axial, front limb, and dyskinesia ratings to produce a dyskinesia score. The dyskinesia score between live and video-based ratings showed good interrater reliability (Lin's coefficient concordance >0.9). A cut-off point of 30 points for the dyskinesia score was chosen as the cut-off point between the low dyskinesia group (n=6) and the high dyskinesia group (n=14) based on K-cluster means analysis. These groups were used for further analysis of behavioral responses.

5.2 Reversal of front-limb use deficit in the 6-OHDA MFB model rat model

The unilateral 6-OHDA MFB lesion decreased the use of the contralateral front limb in the cylinder test (mean 13.5% of all touches, 95% CI 8.3–18.6%, One-Sample T-test p= 0.000025). During the cylinder test, the rats

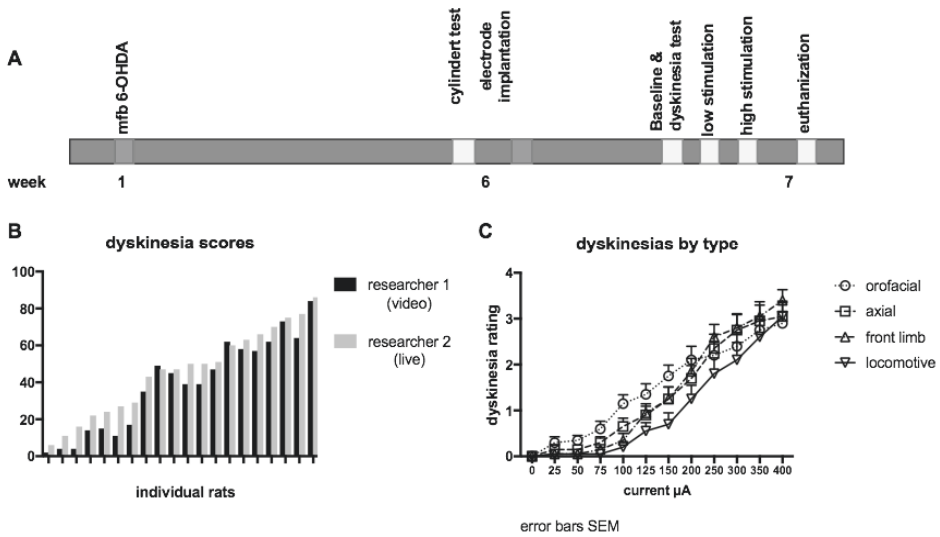

Figure 3 A) study design; B) dyskinesia scores without the orofacial dyskinesias of individual animals, as rated from videos (researcher 1) and live during stimulation (researcher 2); C) stimulation-induced dyskinesias by dyskinesia type across stimulation current (n=18).
Data presented as mean; error bars represent SEM. (submitted to *MethodsX*, to be reproduced under a creative commons license)

reared a median of 10, 17, and 20 times during baseline, low stimulation, and high stimulation, respectively. During these rearing movements, the rats made a median of 46, 63, and 58 contacts, respectively, with the wall with the ipsilateral front limb, 2, 2, and 4 with both front limbs and 9, 21, and 50 contralateral contacts when all contacts with the wall were counted.

As the stimulation currents were chosen based individually on dyskinesia ratings, the stimulation currents in the low dyskinesia groups were higher (low stimulation 103 vs. 200 µA, t-test: $p = 0.002$ and high stimulation 167 µA, 95% CI 138–195 µA vs. 300 µA, 95% CI 243–357 µA, t-test: $p < 0.001$). However, the low stimulation current increased contralateral front limb use in the cylinder test only when all touches with the wall were analyzed and not when only the first touch with the wall was counted. The high stimulation current corrected the contralateral front limb use, as measured by both all and first touches with the wall during rearing movements.

The 6-OHDA-induced deficit of contralateral front limb use was attenuated with STN HFS when compared to baseline without stimulation (low stimulation average 129.0 µA ± 54.2 and high stimulation average 215.0 µA ±90.5). This effect was seen both for first

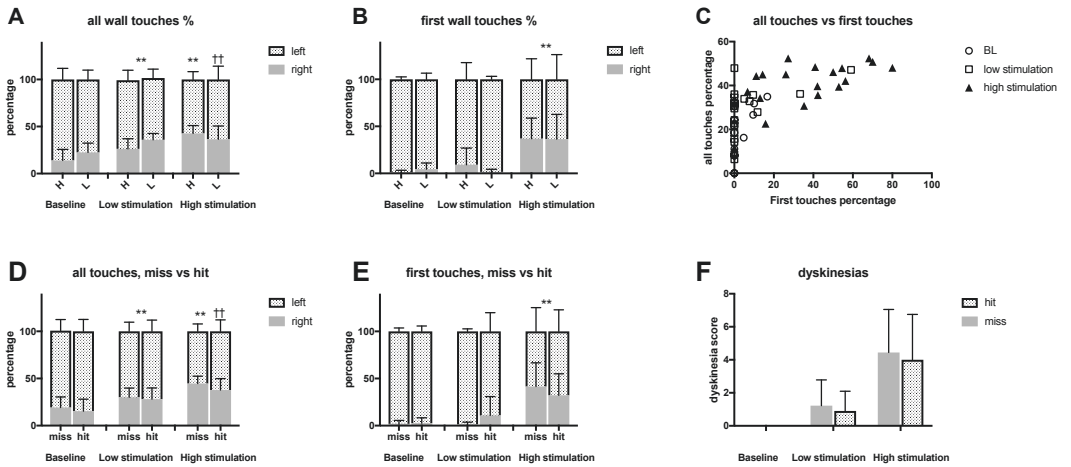

Figure 4 STN HFS with both low and high stimulation reduced front limb use asymmetry in the cylinder test. A) A dose-response effect of STN HFS on contralateral front paw use was observed when counting all front paw touches with the cylinder wall made during rearing movements. B) When counting only the first front paw touches with the wall during rearing movements, improvement in contralateral front paw use was observed only with high stimulation. C) A positive correlation was observed between measurements of contralateral front paw use obtained by counting all touches (x-axis) and only first touches (y-axis) with the wall during rearing movements. D–E) No difference was observed in the reduction of front limb use asymmetry between good electrode placements (hits) and misses, either when counting all touches (D) or first touches (E). F) No difference in dyskinesia severity was observed between good electrode placements and misses at the stimulation currents used in the cylinder test. Data presented as mean; error bars represent SD. H = rats with high dyskinesia score; L = rats with low dyskinesia score

** different from baseline: $p < 0.01$ Bonferroni corrected post-hoc †† different from low stim: $p < 0.01$ Bonferroni corrected post-hoc

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touches with the wall (ANOVA F (2,56) = 22.70, p < 0.001) and all touches on the wall (ANOVA F (2.56) = 25.85, p < 0.001). Increasing stimulation amplitude showed a dose-response relation better the improvement of the contralateral front limb use with higher stimulation currents (Figure 4A-B). The contralateral front limb use in the cylinder test as measured by first touches on the wall and all touches showed good correlation (Spearman's correlation 0.750, p <0.001, figure 4C).

No significant difference was found in front limb use in the cylinder test between the low and high dyskinesia groups (Figure 4A-B). However, a trend indicated that rats in the high dyskinesia group used the contralateral front limb more often at the higher stimulation amplitude when all touches with the wall were analyzed (mean 41% vs. 31%, t-test p= 0.077). Furthermore, after analyzing electrode locations and classifying them as good anatomical hits or misses (Figure 5A), no difference was noted in the front limb use or the level of dyskinesias found at the stimulation amplitudes used (Figure 4D-F).

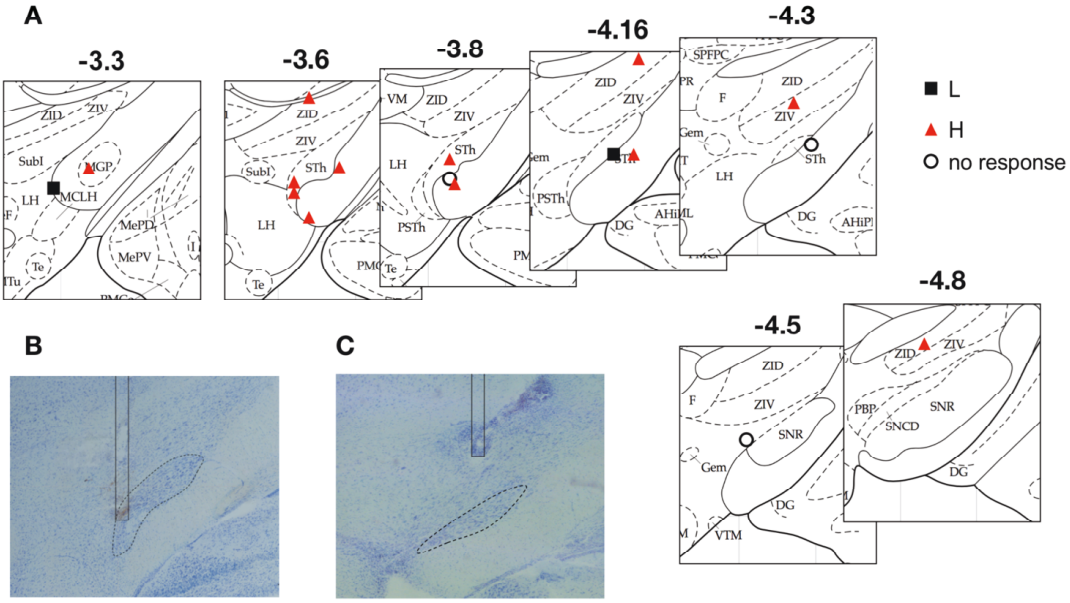

Figure 5 Electrode locations: A) electrode locations in relation to the Paxinos and Watson rat brain atlas with corresponding coronal sections (4th edition, Figures 33-39, used with permission from Elsevier); B) example of a good hit within STN; C) example of a miss, but a good anti-akinetic stimulation effect and high level of dyskinesia.
L = low dyskinesia, H = high dyskinesia, STN/STh = subthalamic nucleus, ZI = zona incerta, dashed line = STN, solid line = electrode (submitted to *MethodsX*, to be reproduced under a creative commons license)
Stimulation-induced dyskinesias recorded during previous dyskinesia-test stimulation at the corresponding stimulation currents in the cylinder test correlated with the improvement of contralateral front limb use (all touches Spearman's r=0.683, p <0.001, 1st touches Spearman's r=0.627, p <0.001) and dyskinesia score and the change of front limb use compared to baseline (all touches Spearman's r=0.584, p <0.001, first touches Spearman's r=0.432, p <0.001; figure 6A-D).

Solving the linear fit equations for all touches

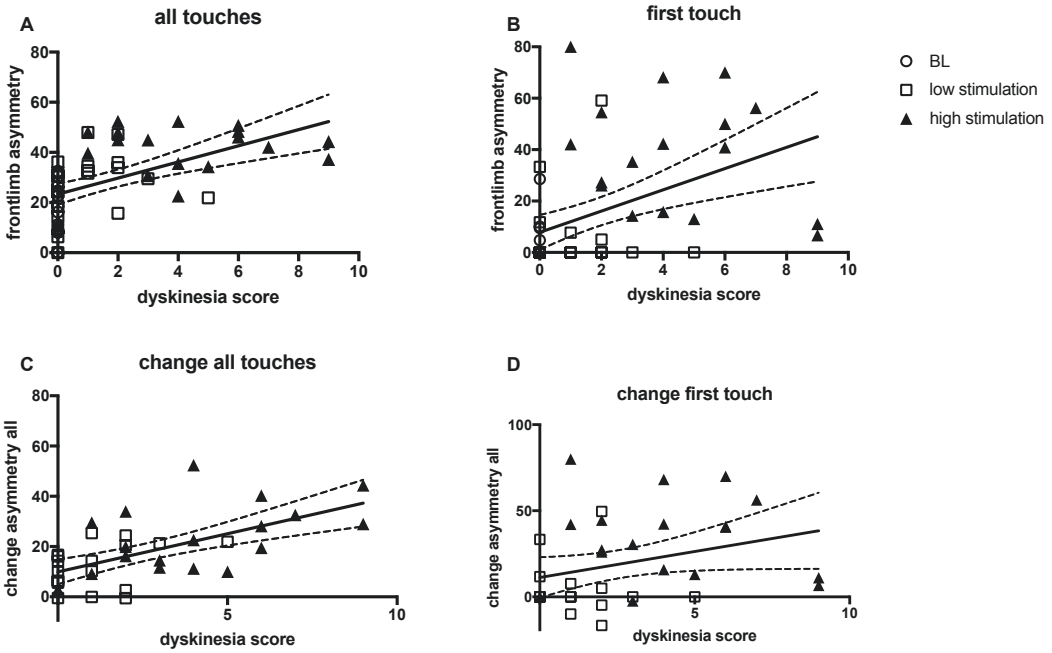

Figure 6. Contralateral front limb use in the cylinder test correlated with dyskinesia scores without orofacial dyskinesia: A) contralateral front limb use as measured by counting all touches with the cylinder wall during rearing movements; B) change/improvement in contralateral front limb use compared to baseline, as measured by counting all touches with the wall; C) contralateral front limb use as measured by counting only first touches with the wall during rearing movements; D) change/improvement in contralateral front limb use compared to baseline, as measured by counting only first touches with the wall. (submitted to *MethodsX*, to be reproduced under a creative commons license)

The relationship between stimulation-induced dyskinesias and improvement of contralateral front limb use deficit was further described by forming and solving linear fit equations of front limb use vs. dyskinesia. Since complete reversal of parkinsonian motor symptoms is not usually feasible even clinically, a cut-off level of 40% was chosen for contralateral front limb use and 25% for improvement of contralateral front limb use to represent the corresponding behaviorally sound improvement of contralateral front limb use deficit. The linear fit equations were solved to determine which level of dyskinesia score, on average, produced the desired improvement in contralateral front limb use. front limb use ($Y = 3.173^{*} + 23.18$, 95% CI 1.874–4.472, $p<0.001$) resulted in a cut-off level of 5.3 (95%CI 3.76–8.96) for front limb use improvement over baseline ($Y = 2.812^{*} + 10.81$, 95% CI 1.447–4.178, $p<0.001$) and in a cut-off level of 5.05 (95% CI 3.4–9.81) for improvement of all touches. For first touches, the linear fit equation ($Y = 4.293^{*} + 7.296$, 95% CI 2.252–6.335, $p<0.001$) resulted in a cut-off level of 4.12 (95% CI 2.79–7,86) and for improvement of front limb use ($Y = 3.23^{*} + 10.89$, 95% CI 1.447–4.178, $p= 0.0326$) the cut-off level was 4.37 (3.38–9.75) for improvement in first touches (Figure 6A-D). The effect of nigral CDNF in MFB 6-OHDA rat model of late PD

The effects of nigral injections CDNF and GDNF were studied with repeated apomorphine-induced rotations (Figure 7A). The number of apomorphine-induced contralateral rotations were similar at 2 and 4 weeks after 6-OHDA injection but before NTF injections between study groups (ANOVA F(6,82)=0.9282, p=0.479 2 weeks and F[6,82]=0.605, p=0.725 at 4 weeks, all post hoc Dunnett tests p>0.7).

A single intranigral infusion of CDNF at four weeks after 6-OHDA lesion at any of the doses (1, 3.3, 10, 33, or 100 µg) did not produce any consistent effect on the drug-induced rotation behavior when compared to the vehicle only (Figure 7B; 6–16 weeks $\chi^2(6) = 13.4–16.3$, p = 0.012–0.038, all post hoc tests p > 0.6, across weeks 6–16 and all CDNF concentrations). A marked reduction in

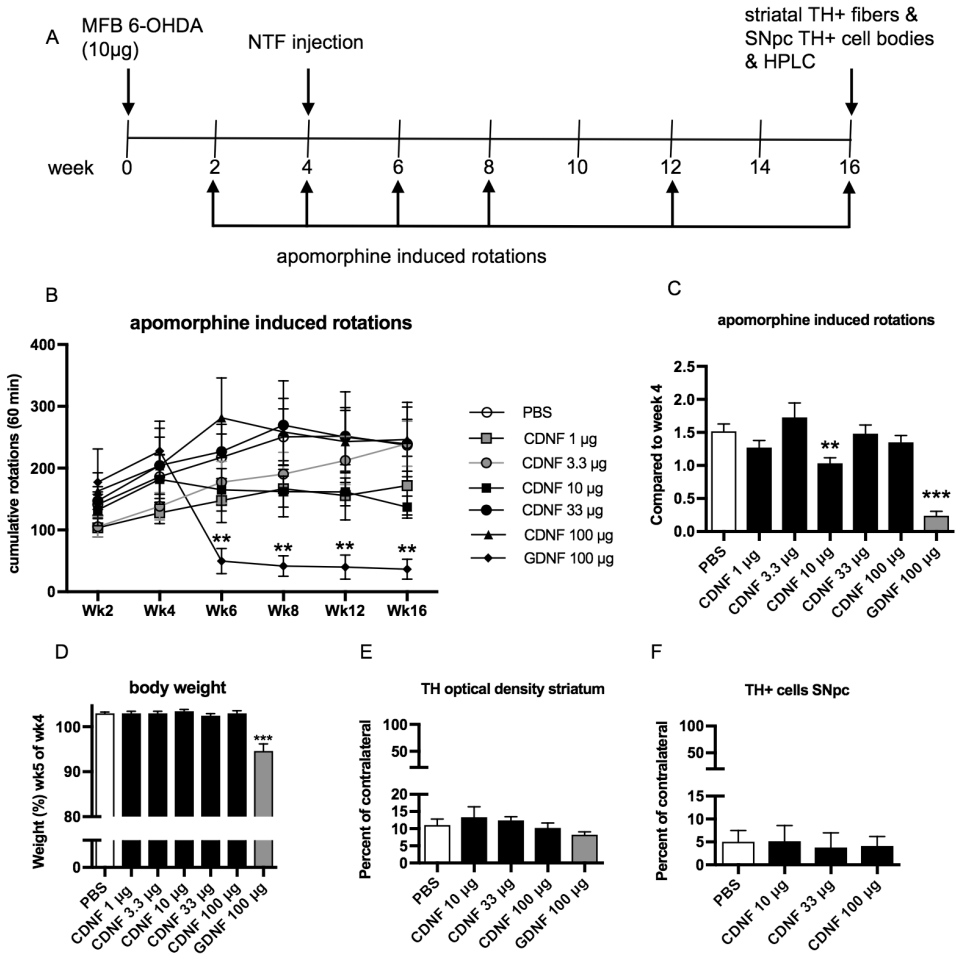

Figure 7. Effects of various doses of intranigral CDNF injection in a 6-OHDA MFB lesion. (A) Study design of experiment 1: NTFs were administered 4 weeks after 6-OHDA. (B) Evolution of apomorphine-induced rotations over time. (C) All apomorphine-induced rotations post-NTF injection compared to rotations at 4 weeks. (D) Average body weight 1 week after growth factor injection compared to body weight before growth factor injection. (E) Tyrosine-hydroxylase-stained striatal optical density of TH+ fibers compared to the contralateral side. (F) Substantia nigra TH+ cell numbers compared to the contralateral side. Data expressed as mean ± SEM, **p < 0.01, ***p < 0.0001. (Reproduced from Huotarinen et al., Neuroscience 2018 under creative commons license)

rotations was seen only with GDNF at 100 µg at all time points after NTF injections when compared to rotations at 4 weeks ($\chi^2(4)$ = 12.65, p = 0.13, post hoc weeks 8, 12, and 16 p = 0.050, 0.008, and 0.020, respectively). At 12 weeks after 6-OHDA injection, GDNF-treated (100 µg) rats also rotated significantly less when compared to PBS-treated rats (Dunnett's test p=0.0493). (Figure 7B)

Analyzing the sum of all apomorphine-induced rotations after the NTF injections, CDNF injections of 1 µg and 10 µg reduced the apomorphine-induced rotations when compared to PBS, although the difference was not significant for 10 µg ($\chi^2(6)$ = 54.69, p < 0.0001, post hoc 0.0307 and 0.10 respectively. When the sum of post-NTF injection apomorphine-induced rotations was compared to weekly rotations at the week 4 baseline before the NTF injection, the apomorphine-induced rotations were statistically significantly reduced for CDNF 10 µg and GDNF 100 µg when compared to PBS (Figure 7C; $\chi^2(6)$ = 63.23, p < 0.0001, post hoc p = 0.0016, and p < 0.0001, respectively).

The GDNF-treated animals experienced a transient reduction in body weight at 1 week after injection (F[6,82] = 18.623, p < 0.001, post hoc GDNF vs. all other groups p < 0.0001, all other post hoc tests p > 0.9) (Figure 7D), but the CDNF-treated animals experienced no loss in body weight with any of the used doses. CDNF (10, 33, or 100 µg) or GDNF (100 µg) did not have an effect on the optical density of TH + fibers in the striatum (Figure 7E, $\chi^2(4)$ = 4.53, p = 0.34). CDNF (10, 33, or 100 µg) did not have an effect on the number of TH+ cells in the SNpc (Figure 7F[3,44] = 0.056, p = 0.982) when NTF was given four weeks after the 6-OHDA lesion.

Similarly, trying to improve the effect of intranigral CDNF 10 µg or GDNF 10 µg by earlier injections at one week after the 6-OHDA MFB did not improve the results. No

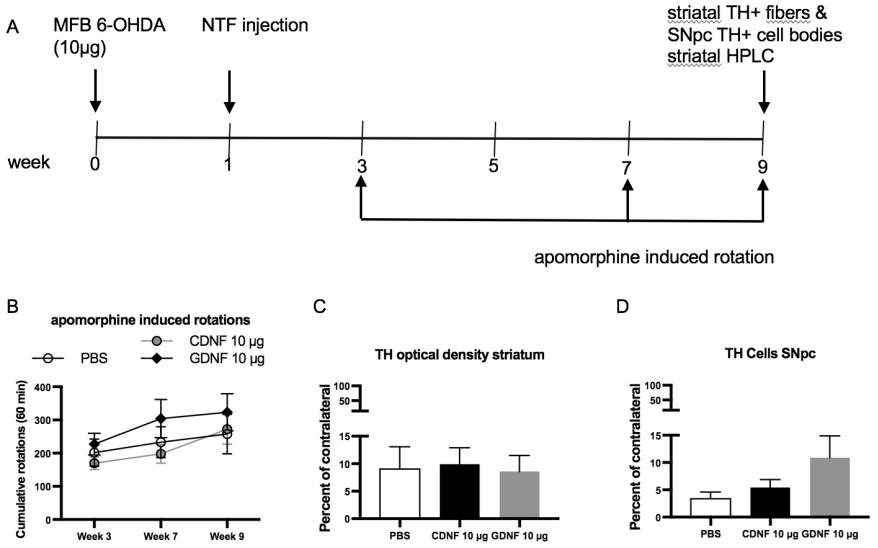

Figure 8: A) Study design of experiment 2 for testing intranigral CDNF 10 µg given 1 week after 6-OHDA. B) Apomorphine-induced contralateral rotations. C) Tyrosine-hydroxylase-stained striatal optical density of TH+ fibers compared to contralateral side. D) TH+ cell count compared to the contralateral non-lesioned site. Data expressed as mean +/- SEM
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decrease was observed in apomorphine-induced contralateral rotations at 3, 7, or 9 weeks when compared to vehicle (Figure 8B; p > 0.50 at all time points). The optical density of TH-stained striata also remained similar with all treatments (Figure 8C; $\chi^2(2)$ = 0.29, p = 0.86) after these earlier injections. The SNpc also showed no difference in TH+ cells (Figure 8D; $\chi^2(2)$ = 2.48, p = 0.29).

5.3 The effect of CDNF on the antiparkinsonian effect of STN HFS

Amphetamine-induced ipsilateral rotations were used to assign the 6-OHDA lesioned rats into similar groups ($\chi^2(3)$ = 1.90, p = 0.59) before CDNF injection and STN electrode implantations. The response to STN HFS was tested repeatedly with cylinder tests (Figure 9A).

The stimulation amplitudes used throughout the study for the control group (PBS + STN HFS) and the study group (CDNF + STN HFS) were: low stimulation 106.3 µA vs. 146.2 µA (U = 130.5, p = 0.030) and high stimulation 181.3 µA vs. 250 µA (p=0.029). For the CDNF +STN HFS group, the stimulation currents were: low stimulation 146.2 µA and high stimulation 250 µA. Both stimulation amplitudes were used on consecutive days after baseline test without stimulation, to test for improvement in front limb use in all rats. Both stimulation amplitudes improved the use of the contralateral front limb in the cylinder test at all time points (p<0.001), except for control group at week 1 ($\chi^2(2)$ = 5.56, p = 0.062) and week 5 ($\chi^2(2)$ = 5.73, p = 0.057).

A cross-sectional analysis comparing CDNF vs. PBS treated animals within individual weeks revealed no differences in the contralateral front limb use in the cylinder tests at any of the time points. However, comparing front limb use at week 2 and 3 to week 1 revealed that only CDNF co-treated rats used the contralateral front limb at these stimulation time points. This effect was not seen with rats receiving PBS in any of the study conditions: no stimulation, low stimulation or high stimulation (Figure 9B-C; t-test week 2 p=0.0603 and p=0.252 week 3; p= 0.0719 and p = 0.0722, respectively, for low and high stimulation). This effect was statistically significant only when week 2 and week 3 time points were analyzed together and compared to week 1 baseline (no stimulation -11.94 SD 22.82 vs. 6.98 SD 19.13, p=0.0073; low stimulation -6.01 SD 25.25 vs. 15.05 SD 23.74 p=0.0125 and high stimulation 20.24 SD 27.17 vs. 40.53 SD 29.45 p=0.0377 respectively). At all time points, the stimulation-induced dyskinesias were similar (Figure 3D, t-test p=0.304–0.908).

The combination of CDNF and STN HFS led to higher levels of striatal TH+ staining when compared to rats treated with PBS and STN HFS (Figure 9E-F, t-test p=0.0347). However, this finding was not further supported by the striatal DAT stainings or nigral counts of TH+ cells (Figure 9G, t-test p=0.251). HPLC also did not show any difference in striatal DA (Figure 9H; ANOVA F(3,14)=0.830, p=0.499)., DOPAC (ANOVA F(3,14)=0.830, p=0.499, 5-HT concentrations or DOPAC/DA ratio (Figure 9I, ANOVA F(3,14)=0.830, p=0.499). No differences were noted in behavioral tests or HPLC analysis in the control rats without implanted electrodes receiving either CDNF or PBS.

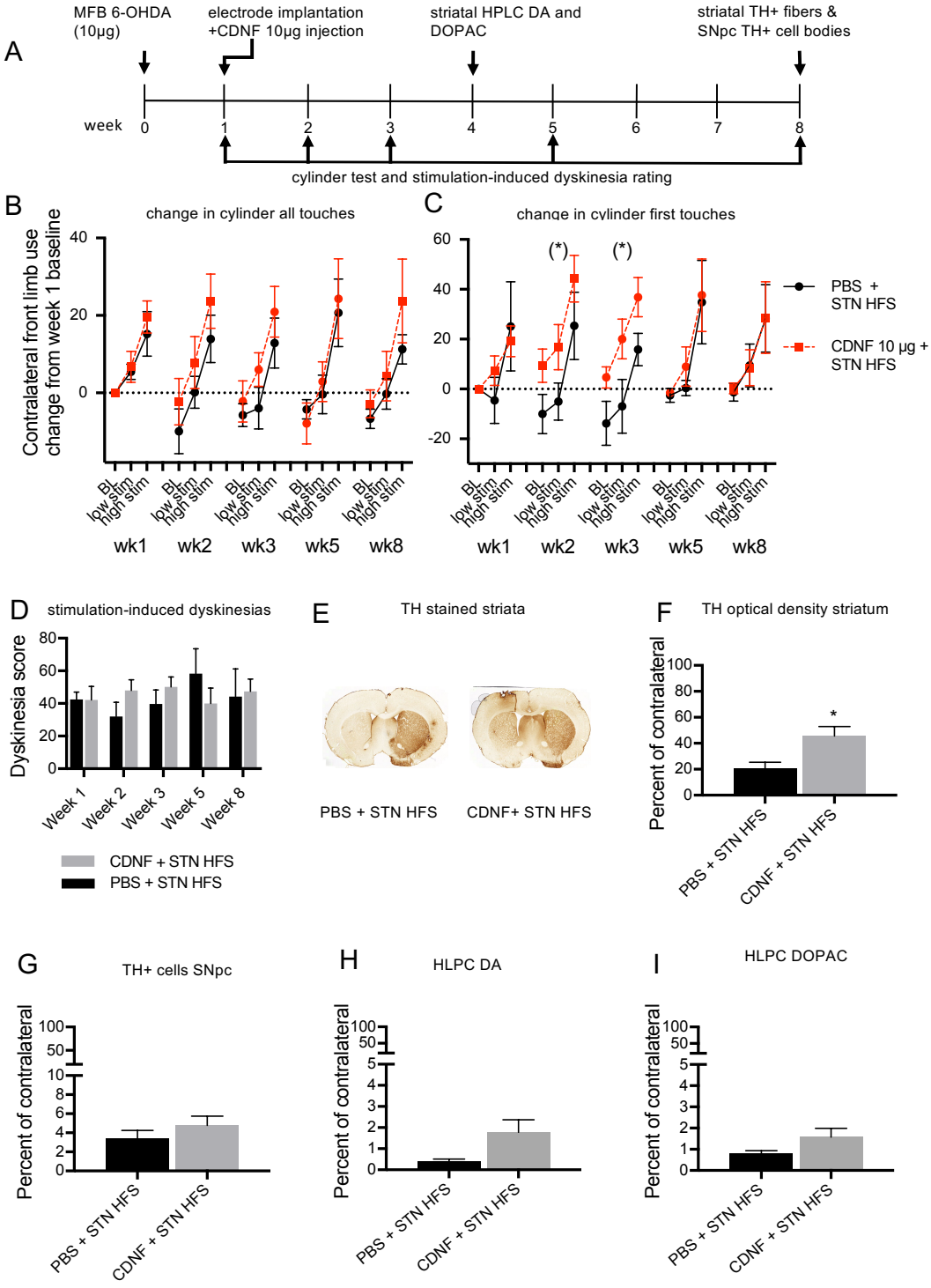
A
MFB 6-OHDA (10µg)
electrode implantation +CDNF 10µg injection
striatal HPLC DA and DOPAC
striatal TH+ fibers & SNpc TH+ cell bodies
week 0 1 2 3 4 5 6 7 8
cylinder test and stimulation-induced dyskinesia rating
B
change in cylinder all touches
Contralateral front limb use change from week 1 baseline
BL low stim high stim
wk1 wk2 wk3 wk5 wk8
C
change in cylinder first touches
(*) (*)
PBS + STN HFS
CDNF 10 µg + STN HFS
D
stimulation-induced dyskinesias
Dyskinesia score
Week 1 Week 2 Week 3 Week 5 Week 8
CDNF + STN HFS
PBS + STN HFS
E
TH stained striata
PBS + STN HFS
CDNF+ STN HFS
F
TH optical density striatum
Percent of contralateral
PBS + STN HFS
CDNF + STN HFS
G
TH+ cells SNpc
H
HLPC DA
I
HLPC DOPAC

Figure 9: The effects of intranigral (left) CDNF 10 µg combined with subthalamic stimulation (STN HFS). A) Study design of experiment 3 for testing the effects intermittent STN HFS combined with CDNF. Cylinder tests were done on consecutive days with no stimulation, low stimulation, and high stimulation. B) The evolution of right limb use asymmetry when all wall touches were counted for the cylinder test compared to baseline (no stimulation) of week 1. C) The evolution of right limb use asymmetry when only the first wall touches were counted in the cylinder test compared to baseline (no stimulation) of week 1. D) Subthalamic stimulation-induced dyskinesias over time. E) Example of TH striatal optical densities at 8 weeks. F) Tyrosine-hydroxylase-stained striatal optical density of TH+ fibers compared to contralateral side at 8 weeks. G) TH+ cell counts in the substantia nigra compared to contralateral side at 8 weeks. H) DA HPLC results at 4 weeks. I) DOPAC HPLC results at 4 weeks. Data expressed as mean +/- SEM, * p<0.05, (*) <0.05 when weeks 2 and 3 were analyzed together. (Reproduced from Huotarinen et al., Neuroscience 2018 under creative commons license)

5.4 The effect of the combination of CDNF and STNL

Amphetamine-induced rotations were used to verify 6-OHDA lesioning and to balance the study groups before the CDNF injections and STN lesioning (ANOVA F[3,52]=0.007, p=0.999).The behavioral effect was tested with repeated cylinder apomorphine-induced rotation tests. (Figure 10A). Ten rats were excluded from the analyses due to macroscopic lesions (1 mm or over) in the subthalamic area or the thalamus.

STN lesioning with ibotenic acid combined with intranigral CDNF reduced apomorphine-induced rotations at 4 weeks when compared to a double sham treatment (PBS + PBS). Neither of the treatments alone had this effect (Figure 10D, F[3,41] = 3.853, p = 0.16, post hoc Tukey for CDNF + STNL vs. PBS + PBS p = 0.014). This effect was not carried over to week 7 time point, although the STNL + CDNF treated animals rotated the least, albeit this was not statistically significant (χ^2 [3] = 6.2, p = 0.10). Similarly, neither of the treatments alone improved the contralateral front limb use over the double sham treatment. By contrast, the combination of STNL and CDNF improved contralateral front limb use in the cylinder test (Figure 10B-C). This effect was seen at week 1 both when analyzing all touches (χ^2 (3) = 10.16, p = 0.017, post hoc p = 0.047, animals with less than 20 touches on the wall were excluded) and first touches (χ^2 (3) = 11.02, p = 0.012, post hoc p = 0.012) with the cylinder wall. A similar effect was seen at week 3 for all touches (χ^2(3)=15.84, p=0.001, post hoc p= 0.011) and first touches (animals with under 7 rearings excluded, χ^2(3) = 9.28, p = 0.026, post hoc p = 0.032) with the wall. At week 6, the number of rearings was low and first touches with the cylinder could not be analyzed, but a tendency was evident for the CDNF + STNL co-treated rats to use the contralateral front limb more than the rats that received intranigral CDNF and sham STNL when all touches with the wall was analyzed (PBS) (χ^2(3) = 6.52, p = 0. 089).

When the use of contralateral front limb use in the cylinder test was compared to baseline before intranigral and subthalamic injections, only the group of rats that received the combination treatment improved at any of the time points. This was significant for all touches at week 3 (Friedman's χ^2 = 9.171, p = 0.027, post hoc p < 0.001), all other p-values >0.2) and for first touches at weeks 1 and 3 (Friedman's χ^2 = 13.91, p = 0.001, post hoc p = 0.093 and 0.002, week 6 excluded from analysis due to the low number of rearings).

Because some of the rats were excluded from the analyses due to inactivity in the cylinder test or because of macroscopic STN

region lesions, the data were reanalyzed with all animals included, and the same effects were seen. The only major change was that the decrease in apomorphine-induced rotations at week 4 did not reach statistical significance in the post hoc test for CDNF + STNL treatment over double sham treatment when comparing all the study groups against each other ($F[3,50] = 2.211$, $p = 0.087$, post hoc Tukey $p = 0.085$, Dunnett's $p = 0.049$).

The immunohistochemical analysis revealed no difference between the study groups at 8 weeks after intranigral and subthalamic injections. The TH stained striatum optical density showed no difference (Figure 10E; $\chi^2(3) = 4.347$, $p = 0.226$) nor did the TH+ cell count in SNpc (Figure 10F; $\chi^2(3) = 0.51$, $p = 0.92$). Striatal dopamine (Figure 10G; $\chi^2(3) = 2.04$, $p = 0.57$) and dopamine metabolite levels were also similar (DOPAC Figure 10H; $\chi^2(3) = 2.97$, $p = 0.40$). The levels of striatal 5-HT concentrations and DOPAC/DA ratios were similar.

Figure 10: A) Study design of experiment 4 for testing the combination of intranigral (left) CDNF 10 µg and STN lesion (STNL). B) Front limb measured by all touches with the wall during rearing movements in the cylinder test at baseline before CDNF injections and STN lesioning and at 1, 3, and 6 weeks after. C) Front limb measured by first touches with the wall during rearing movements in the cylinder test use at baseline before CDNF and STNL and at 1, 3, and 6 weeks after. D) The number of apomorphine-induced rotations at 4 and 7 weeks after CDNF and STNL. E) Optical density of TH immunohistochemistry of lesioned side compared to the unlesioned side. F) The number of TH+ cells in SNpc compared to the unlesioned side. G) HPLC results for percentage of dopamine (DA) on the lesioned side compared to the unlesioned side. H) HPLC results for percentage of DOPAC on the lesioned side compared to the unlesioned side. Data expressed as mean +/- SEM, * $p<0.05$, ** $p<0.01$
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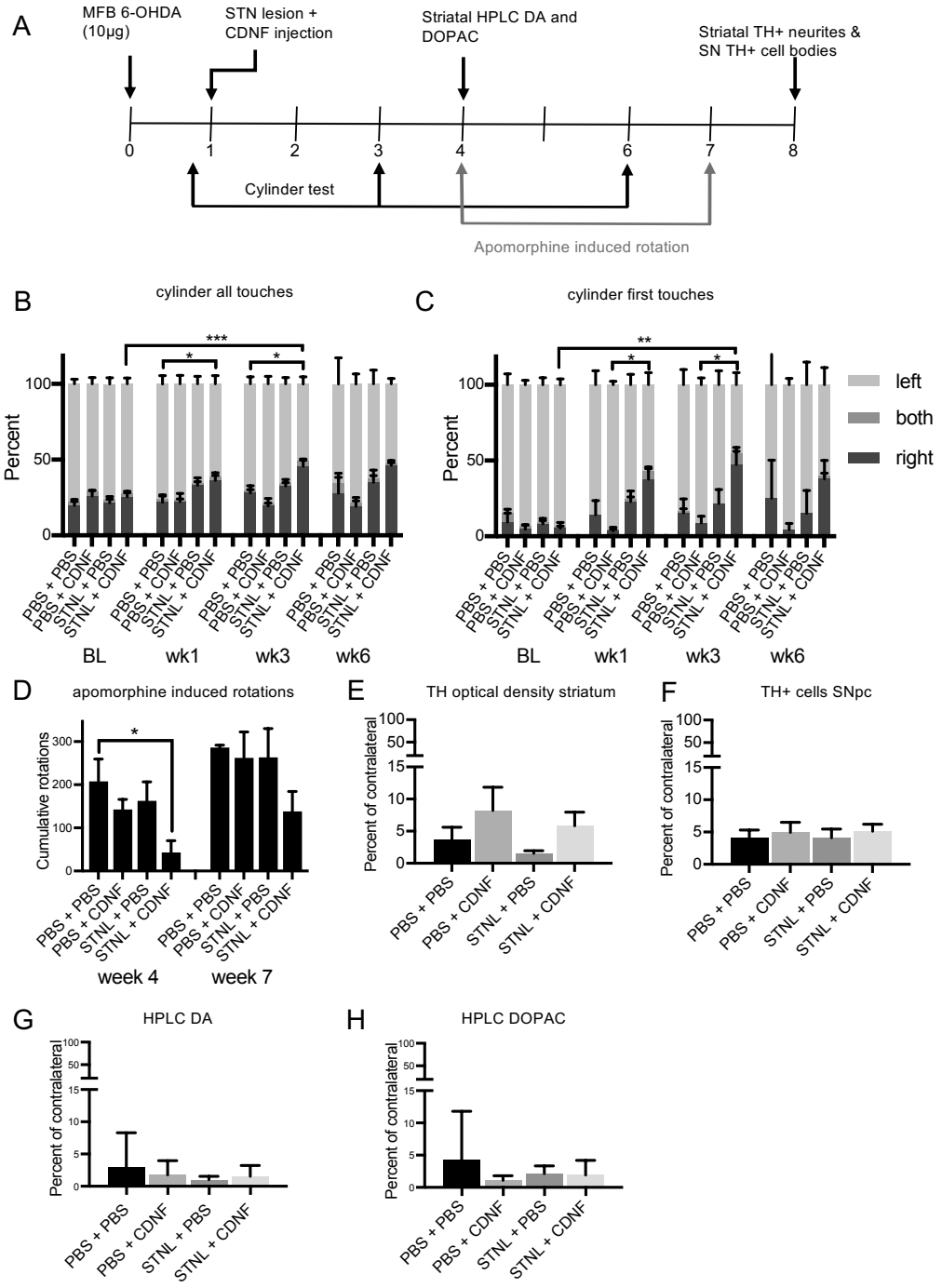
A
MFB 6-OHDA (10µg)
STN lesion + CDNF injection
Striatal HPLC DA and DOPAC
Striatal TH+ neurites & SN TH+ cell bodies
0 1 2 3 4 6 7 8
Cylinder test
Apomorphine induced rotation
B
cylinder all touches
C
cylinder first touches
Percent
left
both
right
PBS + PBS
PBS + CDNF
STNL + PBS
STNL + CDNF
BL
wk1
wk3
wk6
D
apomorphine induced rotations
Cumulative rotations
week 4
week 7
E
TH optical density striatum
F
TH+ cells SNpc
Percent of contralateral
G
HPLC DA
H
HPLC DOPAC

5.5 The effect of clinical STN DBS

During the study period, the annual number of DBS operations for PD increased after 2010, reaching 41 operations in 2014. The total number of patients with PD who underwent STN DBS was 103, with 206 electrodes implanted as all operations were bilateral. After exclusion of patients with incomplete clinical data and insufficient imaging quality, 87 patients (53 male, 43 female) were included in the final analysis. During the time of DBS surgery, the median age was 61.0 (IQR 54.0–65) years. The time from PD diagnosis to DBS surgery was a median of 11 years (IQR 8–15). The most common indication for STN DBS was L-DOPA-related motor fluctuations or dyskinesia (85%). Other indications were drug resistant tremor (13%), other L-DOPA related side effects, or severe dystonia (2%).

At six months after STN DBS, the severity of PD motor symptoms on the UPDRS-III scores was reduced by 15.9 ±12.7 in the stim ON med OFF state when compared to pre-DBS med OFF state (38% reduction, Wilcoxon signed rank test, p<0.001). The disease severity showed a statistically significant reduction as measured by the H&Y score (Wilcoxon signed rank test, p=0.005) from 2.3 med OFF before STN DBS to 2.2 stim ON med OFF. Although this reduction of 0.14 points was statistically significant, the absolute reduction was too small to represent a clinically significant reduction, unlike the UPDRS-III reduction. (Table 3)

The use of dopaminergic medication, as measured by LED, decreased at six months follow-up from a median of 1117mg (IQR 793–1451 mg) to 670 mg (IQR 407–1087)). However, a large variability was evident in individual changes in dopaminergic medication use, ranging from a decrease of 1500 mg to an increase of 751 mg in LED. At six months, five patients (6%) were completely weaned off of L-DOPA.

Table 3. The UPDRS part III scores, LED changes, H&Y scale changes, and the mean stimulator settings. Results presented as mean and interquartile range from 25% to 75% *UPDRS-III =Unified Parkinson's disease rating scale part II, LED = levodopa equivalent dose; H&Y= Hoehn and Yahr stage.*

	Pre-DBS	6 months	p-value
UPDRS-III (med OFF)	37 (31–48)	22 (IQR 17–29)	<0.001
H&Y (med OFF)	H&Y 2.5 (2.0–3.0)	2.0 (IQR 2.0–2.5)	0.005
LED mg	1117 (793–1451)	670 (407–1087)	<0.001
Amplitude V		2.3 (1.9–2.6)	
Pulse width µs		60 (60–60)	
Frequency Hz		130 (130).	

Bilateral monopolar stimulation was the most common stimulation configuration (72% of the patients), followed by bilateral bipolar (14%) stimulation, and patients having bipolar on one other side and monopolar on the other (14%). Four patients (5%) had adjacent monopolar electrodes as active electrodes. Additionally, two patients had interleaved stimulation. The mean stimulation parameters were: amplitude 2.8 V (range 1.7–4.85 V), pulse width 60 µs (range 60–90 µs) and frequency 130 Hz (range 60–180 Hz). In three patients, a constant current mode was used instead of a constant voltage mode. Two or more stimulation programs were made for 29 patients, but only 5% of them trialed these additionally available programs, according to usage data stored in IPG. The additional programs allowed for the possibility of using different stimulation frequencies (82%) or pulse widths (7%), or changing the active electrode contacts (11%).

5.6 Adverse effects of STN DBS

Operation or hardware related complications were experienced by 16 (18.4%) patients. Infections were the most common of these complications: fourteen patients had superficial skin infections treated with antibiotics, nine patients underwent superficial revision surgeries for these infections, and one IPG was removed due to infection. One patient had a symptomatic intracerebral hemorrhage with good recovery. Chronic subdural hematoma was found in one patient one month after the DBS operation without other apparent reasons apart from the operation. Additionally, in four patients, the electrodes passed through ventricles without apparent side effects. Dysarthria was the most common patient-reported stimulation-related side effect found in 22 patients (25%). Nevertheless, four patients (5%) showed an increase of only 2 points in UPDRS-III item 18 (speech), which was regarded as significant worsening, and this item increased by only 1 point in an additional 14 patients. Transitional confusional state was seen in six cases, a brief episode of psychosis in one case, and severe depression in one case. One patient committed suicide during follow up and one patient attempted suicide. Difficulties in maintaining balance were reported by seven patients.

5.7 The relationship between different methods of electrode location analysis and the relation of electrode location to the clinical effect

The coordinates acquired with different methods showed statistically significant correlations with each other (Spearman correlation coefficient 0.48–0.82, all correlations significant at $p<0.001$). The median locations of electrodes and the interquartile range (IQR) are shown in Table 4**.** Overall, the placement of the center active electrodes was in the subthalamic area in the vicinity of STN, with approximately half of the

Table 4. Median locations of the electrodes acquired with three different methods.
Atlas coordinates: = lateral from midline, Y = posterior from AC, Z = inferior to AC, nucleus ruber (NR) coordinates: = lateral from lateral border of NR, Y = posterior from anterior border of NR, Z = inferior from superior border of the NR; MCP (midcommissural point) coordinates: = lateral from midline, Y = posterior from MCP, Z = inferior from MCP. All coordinates in mm. (Koivu, Huotarinen, et al.[350] *Brain and Behavior*, reproduced under a creative commons license)

	Atlas		Nucleus Ruber		MCP	
	Median	IQR	Median	IQR	Median	IQR
Right	11.1	10.3–12.4	3.0	2.3–3.9	12.0	11.1–12.9
Right Y	17.2	16.0–18.6	0.1	-1.3–1	3.4	2.1–4.3
Right Z	4.9	4.1–5.8	1.2	0.0–2.3	3.2	2.0–4.6
Left	10.3	9.1–11.2	1.8	1.0–2.9	10.1	10.1–11.9
Left Y	17.2	16.9–18.6	0.1	-0.9–1	3.1	1.9–4.3
Left Z	4.9	3.7–5.8	1.0	0.0–2.1	3.0	1.5–4.4

electrodes having the center within the borders of the STN based on visual inspection. An overall tendency was noted for the electrodes to be located slightly medio-dorsally to the STN.

The differences in the electrode locations in relation to LED reduction of over or under 30% are shown in Table 5 for the nucleus ruber (NR) coordinates. The patients with greater LED reduction had more ventrally located electrodes in relation to the nucleus ruber coordinates (Mann Whitney U-test=0.025) and MCP coordinates (Mann Whitney U-test=0.007), but not in the atlas-based coordinates (Mann Whitney U-test=0.20). No statistically significant differences were found for the electrode location in patients who had over or under 30% improvement in UPDRS-III at 6 months (Mann Whitney U-test values 0.059–0.975 for the correlations in different coordinate systems).

Table 5. Patient groups and electrode coordinates in relation to the nucleus ruber (NR) coordinates. Median electrode location corresponds inferomedial part of the subthalamic nucleus (STN). STN location in relation to NR as measured from the Mai Atlas is: = 1.9-8.3 mm, Y = -3.9 – 2.7 mm, Z = - 1.8 – 1.8 mm. NR = nucleus ruber, n = number of patients, IQR=interquartile range, * Sig<0.05 statistically significant using Mann-Whitney U test. LED = Levodopa equivalent dose, = medio-lateral direction, Y = antero-posterior direction, and Z = dorso-ventral direction (Koivu, Huotarinen, et al. [350] *Brain and Behavior*, reproduced under a creative commons license)

LED DECREASE AT 6 MONTHS		**RIGHT**			**LEFT**		
	in relation to NR	**X**	**Y**	**Z**	**X**	**Y**	**Z**
<30%	Median	3.0	0.0	1.1	1.7	0.0	0.6
	N	44	44	44	44	44	44
	IQR	2.0–3.9	-1.6–0.7	0.0–2.6	2.2–6.6	-1.2–1.0	-.08–1.7
≥30%	Median	3.0	0.5	1.4	2.1	0.0	1.6
	N	43	43	43	43	43	43
	IQR	2.5–3.8	-0.9–1.0	0.4–2.3	1.0–3.0	-0.6–1.1	0.3–2.3
	Sig.	0.644	0.099	0.131	0.231	0.085	**0.025***

6 DISCUSSION

6.1 The dyskinetic and anti-akinetic effects of experimental STN HFS

An increase in stimulation current increased the occurrence of stimulation-induced dyskinesias, but a better reversal of 6-OHDA induced front limb use deficit, indicating a dose response of STN HFS. While the order of appearance of stimulation-induced dyskinesias has been previously reported,[322] a gradual increase in intensity has not been described. Another observation was that achieving a significant reversal of front limb use deficit required a sufficiently high stimulation current to cause transient dyskinesias, as shown by the correlation of front limb use and stimulation-induced dyskinesias. This feature can be used to select suitable individual stimulation amplitudes for STN HFS. The initial stimulation-induced dyskinesias could also predict good therapeutic effects of clinical STN DBS.[226] The gradual appearance of stimulation-induced dyskinesias seems also to suggest that a grading system, such as the one presented here, could be more reliable for choosing individual stimulation currents when compared to the singular appearance of dyskinesia threshold used in some previous studies[322]or the same current used for all animals.[351] The use of orofacial dyskinesias seemed not to have any additional benefit and these dyskinesias were also difficult to score reliably from videos. Orofacial dyskinesias have also been suggested to be mediated by different mechanisms than other dyskinesias.[322,352] This suggests that orofacial dyskinesias should perhaps be excluded from the analysis of STN HFS-induced dyskinesias or they should be analyzed separately. Occasionally, STN HFS also induced hind-limb and, rarely, ipsilateral front-limb dyskinesias, which could be explained by somatotopy of the STN and may therefore be related to the exact electrode location in the subthalamic region.[353] The stimulation-induced dyskinesias seen in experimental STN HFS do not necessarily replicate the situation in clinical STN DBS, because of cross-species differences in anatomy, electrode configuration, and stimulation timespan.

Interestingly, although front limb use in the cylinder test measured by first and all touches with the cylinder wall showed good correlation, the beneficial effect on the first touches with the wall was seen only with the higher stimulation amplitude. This suggests that the first touch with the wall is a more robust phenomenon, perhaps reflecting the movement initiation deficit related to dopamine depletion and PD. However, the improvement in front limb use when all touches with the wall were analyzed even with the lower stimulation amplitude suggests that this is a more sensitive measure. Using both methods during analysis of the cylinder test is supported by our data as this will allow acquisition of a more robust and sensitive measure when analyzing experimental treatments, as opposed to using only one or the other, as has been done in some previous studies.[106,351] An additional benefit of using both measures might be seen during repeated cylinder tests, as some rats become less active and rear less and other rats might be so inactive that the use of only the first touches becomes statistically unreliable because of the low number of observations. Some of these rats could still be included in the analysis by using all touches with the wall to analyze the front limb use, thereby reducing the number of animals required. Since changes in the front limb use during stimulation with the lower amplitude were seen only when analyzing all touches, our data suggest using both measures to ensure that subtler effects are not missed. An additional benefit of developing the analysis of cylinder test data

is that the cylinder test does not require pharmacological stimulation, such as the apomorphine or amphetamine used in the rotation test. The reversal of front limb use in the cylinder test reflects reversal bradykinesia, as is seen in clinical PD with STN DBS

All the electrodes were found in the subthalamic area in the STN or in the immediate vicinity and no difference was observed in the level of stimulation-induced dyskinesias or the reversal of front limb use deficit. The stimulation current spreads from the active electrode contact to the adjacent brain tissue, where a 1 mm distance exists between the two contacts in the electrode. This suggests that the zona incerta (ZI) stimulation might explain the good effect achieved with electrode tips outside the STN, as also clinical benefit has also been reported following ZI stimulation. Some clinical studies also suggest that the ZI might be a better target than the STN for DBS for PD.[255] These findings support that animals in the experimental setting should not be included in or excluded from the final analysis based only on behavioral data or electrode tip anatomic location; instead, both measures should be taken into account.

6.2 Intranigral CDNF in a late-stage PD model

In the rat MFB 6-OHDA model of advanced PD, which produces a very severe dopaminergic lesion,[354] no consistent reduction was observed in apomorphine-induced rotations by intranigral CDNF alone given at various doses 4 weeks after 6-OHDA injection or by 10 µg CDNF given one week earlier after 6-OHDA injection. The trial replicated the results of a very large dose of 100 µg GDNF by Hoffer et al.[355] without concomitant increase in striatal TH.[356] Nevertheless, a small putative effect of 10 µg CDNF was revealed by analyzing all the post-NTF apomorphine rotations together, but this did not correlate with rescue of TH positive neurites in the striatum or TH positive cells in SNpc. Interestingly, treatment with the 100 µg GDNF led to a transient decrease in body weight,[355,357,358] suggesting an overall negative effect of GDNF on animal welfare when compared to CDNF, which caused no drop in body weight at any of the trialed doses.

Unlike in previous studies using less severe models and mainly striatal NTF injections,[359-361] no effect was observed when CDNF alone was given intranigrally. This is in line with the fact that the striatum is almost completely devoid of TH positive neurites 5 years after PD diagnosis[11] and, correspondingly, some neuroprotective effect has been reported only in patients with disease durations of less than five years.[15] NTF-induced neuroprotection is suggested to require viable dopamine neurons in the SNpc.[362] However, since the accuracy of the initial PD diagnosis is low,[38] a follow-up period of at least 5 years has been suggested before invasive treatments to rule out patients with atypical PD. [12] More recently, a recommendation was made for consideration of invasive treatments earlier in the course of disease.[201] The lack of a good effect could be explained by the choice of an intranigral injection site, since striatal injections have previously been found effective. However, striatally injected CDNF is also transported to the SNpc.[363] The poor results in the clinical trials might be explained by insufficient diffusion[363] of NTFs in the larger human striatum, as an increase in TH-positive fibers has been found around the injection sites in post-mortem samples.[364] This implies that, despite our mostly negative results with intranigral injections, the SNpc might have some favorable aspects as an NTF injection target as it is smaller and easier to cover with even relatively weakly diffusing agents.

6.3 CDNF and acute STN HFS

In the 6-OHDA MFB model, STN HFS ameliorated 6-OHDA-related motor deficits in rats that received intranigral injection of either PBS or CDNF. Despite the lack of a statistically significant difference in contralateral front limb use when comparing the groups at respective weeks, the effect of STN HFS was transiently increased at two and three weeks after CDNF injection when compared to the first week after implantation. A tendency to improve tyrosine hydroxylase levels in the striatum was also evident after 8 weeks, which, disappointingly, was not statistically significant and was not replicated by an increase in DAT. However, behavioral NTF effects might not completely depend on the restoration of dopaminergic terminals or neurons.[365] These putative changes in front limb use seen in the cylinder tests might reflect the potential of similar combinations of CDNF and STN DBS for added benefits in treatment of bradykinesia.

6.4 Combined CDNF and STNL

The combination of a STN lesion and CDNF alleviated parkinsonian motor deficits, as measured by two different behavioral tests and the effects were not seen with either treatment alone. No clear biochemical effect was seen in the striatal density of tyrosine hydroxylase-positive fibers or nigral TH-positive cells or dopamine and its metabolites, but a trend was evident for an overall CDNF effect on striatal TH levels. Previous reports have shown that CDNF can produce a partial reversal of the behavioral defects in the 6-OHDA hemiparkinsonian rat without changes in TH or monoamine transmitter levels[365] and that the correlation between IHC-verified striatal TH-deficits and apomorphine-induced rotations is low.[66]

A positive interaction is evident in the behavioral tests. Apomorphine-induced rotations provide a crude measure of a possible benefit, but the behavior is produced by artificial pharmacological stimulation, so it might not reflect the situation in clinical PD. However, a similar result was seen with the cylinder test, which provides a more natural and also a more clinically relevant measure of the beneficial effects of reducing bradykinesia. The results should be viewed as preliminary and require verification in different PD models, and with other behavioral tests, preferably also by other groups and with other NTFs. The effect might be neuroprotective or neurorestorative, or, alternatively, the combination might also act solely on a level of symptom control. Although our experiments did not address the mechanism of the effect, previous studies have shown a synergy for the combination of CDNF and GDNF[332] or MANF[333], and the NTF-like properties of trkB signaling[292] and BDNF induction[324] might explain some of the perceived effects.

These data show only a transient effect of a single infusion of CDNF, whereas clinical trials have used either AAV constructs[366] or chronic administration of NTFs[367]. Additionally, STNL and STN HFS have both shared and distinct effects[281]. Unfortunately, the observed effects seen in the present study were of relatively short term, so future studies should assess the effects of longer term CDNF treatments in combination with DBS models.

In clinically advanced PD, the prospect of neurorestoration is quite low[49], so the fact that the synergistic effect does not rely on the regeneration capability of dopamine system may be beneficial. Our results this suggest that NTFs delivered to the SNpc, together with STN DBS, might have additional benefits as a treatment for clinical PD. However, additional animal experiments are needed before planning clinical studies to investigate this combination.

6.5 Clinical results of STN DBS

The results presented here for clinical STN DBS are comparable to those of earlier studies,[240] with a reduction in UPDRS-III scores of 40%. However, Herzog and Schüpbach have reported decreases of 51–53% [199] and even close to 70% at 18 months.[245] The reduction in dopaminergic medication use measured by LED was also significant and similarly in line with previous studies.[240,368] Our patients showed fewer neuropsychiatric side effects;[369] however, since no routine neuropsychological testing was conducted, some cases with mild neuropsychiatric side effects might have been missed. Previous studies have found that neuropsychiatric side effects were related to a more ventral location of the active electrode,[256] but this was not supported by our data. The most common stimulation-related side effect was dysarthria, which is a previously known troublesome side effect of STN DBS.[370-372] The incidence of intracerebral hemorrhages and post-operative infections was similar to previously described occurrences.[373]

6.6 Electrode location analysis and optimal target

The different methods of acquiring electrode locations gave similar but not perfectly correlated results, supporting previous publications that found different validity for different coordinate acquirement methods. Previous studies reporting on the direct visualization of STN have used 3T MRI, [217,347] as 1.5T MRI does not provide as good STN visualization.[374] We used 1.5 T MRI, but our data still show a correlation between direct and nucleus ruber and MCP-based coordinates.

Unlike previously reported findings[255], our patients with better outcomes had more ventrally placed electrodes, although this was not evident from direct visualization of atlas-based coordinates. However, a tendency was indicated for the average location to be slightly medial to the STN, which might lead, in effect, to stimulation of the structures along the dorsal border of the STN. According to a recent survey, most specialists in the field place the optimal target for STN DBS either inside the STN or on the dorsal border.[8] Our finding might indicate that better results could be achieved with intra-STN positioning, but, surprisingly, we did not find any increase in neuropsychiatric side effects as was reported for a large retrospective follow-up study.[256] We also found good responses to STN DBS within a larger area, which might also suggest that other factors than electrode position have strong influences on the therapeutic effect. However, the anatomical spread of the electrode location was relatively large, which, together with the pooling of both sides for analysis, might affect the reliability of the present statistical analysis. The fact that we used 1.5T and control CT scans taken 1–2 days postoperatively might also affect the accuracy of the electrode location analysis[375], although the amount of intracranial air was small.

6.7 Future Prospects

Both the experimental and clinical STN stimulation (HFS and DBS) can alleviate parkinsonian motor deficits, but the reversal of symptoms is not complete and not without side effects. In pre-clinical animal studies, the effect on motor deficit is often studied using only one stimulation current. Our results suggest that, in some instances, different stimulation amplitudes should be used. Our experimental results also suggest that both behavioral and electrode location data should be used in future studies when selecting animals for final analyses. Although many previous studies have used a standard

stimulation amplitude for all animals in the experiment, the high variability of individual responses better supports the use of individually tailored stimulation amplitudes. However, since the optimal target for animal STN stimulation is not completely known, conducting studies that compare small variations in the subthalamic targeting with various electrodes would be reasonable, provided the accuracy of animal stereotaxy used is precise.

Similarly, despite considerable previous research, the optimal target for clinical STN DBS is still under debate. The continuous improvements in imaging methods, operating techniques, and stimulation hardware will ensure that this question will continue to be an interesting, if controversial, research subject for the foreseeable future. Our data show a positive treatment response coming from a large subthalamic area, which also suggests that future clinical studies should report the electrode locations, as this is disappointingly not often done. One of the major limitations of the clinical data is that electrode locations were defined using almost immediate postoperative imaging, which is subject to error. This encourages the use of either delayed CTs at one month or later or the more widely available intraoperative MRI to achieve higher precision in electrode location definition. Additionally, the analysis of clinical data highlights the problems of statistical analysis of electrode locations. The effect of electrode location on motor symptoms and side effects could be better analyzed if the statistical methods used allow for analyses in 3D spaces or at least at the distance of the electrodes to neuroanatomically meaningful structures and not simply the orthogonal axes of the stereotactic space.

Furthermore, neither STN DBS nor any other currently widely available therapies can alter the course of clinical PD. NTFs are among therapies being studied to provide a disease-course altering therapy in PD. However, as discussed above, in practice, these therapies seem often to be implemented in clinical situations where the degeneration of the dopamine system has progressed so far that the possibility for neurorestoration seems low. One solution for this is the suggestion that both DBS and NTF therapies be offered earlier in the course of the disease, and this is supported at least in part by the data presented here. Additionally, myriad possible reasons exist for the previous failures of clinical NTF trials. One reason, without question, is the fact that, as in our experiments, the experimental PD is often based on neurotoxin models that do not reflect all aspects of clinical PD. The use of a amore biologically valid α-synuclein model with LB and LN accumulation could provide a better testing ground for future neuroprotective and neurorestorative therapies. In addition, the customary protocol is to test promising disease-course altering therapies in models with relatively modest levels of dopamine degeneration. This can perhaps explain some of the disappointments in clinical trials and future studies should strive to achieve experimental neuroprotection and neurorestoration in animal models that are compatible with advanced PD where, in general, a practical possibility exists to use invasive treatments.

Another possibility is that nearly complete loss of the dopamine phenotype impairs the possibility of neuroprotection and neurorestoration altogether. This implies that achieving success in the search of disease-course altering therapies will require reliable early diagnosis of neurodegenerative diseases. For PD, neuroprotection could possibly be achieved in the premotor phase of the disease, when the dopamine system is still quite viable. Together with previous data, our results suggest that successful neuroprotection in PD requires the use of

several strategies in experimental studies, clinical studies, and timing of treatments. Especially in the more advanced phases of PD, no single treatment alone is likely to provide a complete solution. Therefore, combinations of neuroprotective/restorative and symptomatic treatments should be studied together, both preclinically and clinically.

7 CONCLUSIONS

The main objectives of this work were to further describe the effects of STN DBS in clinical PD, to compare different methods of analyzing electrode location, to further analyze the behavioral response of STN DBS in an animal model, and to study the effect of the combination of CDNF and STN DBS in an experimental animal model of PD that shows dopaminergic degeneration levels corresponding to late-stage real-world human patients.

The main conclusions are:
1) Correct electrode placement should be defined both behaviorally and histologically in rat STN DBS. Tailoring the behavioral response quite effectively is possible using individually chosen stimulation amplitudes in experimental studies. Conducting behavioral tests with several amplitudes can improve the sensitivity and reliability of the analysis.

2) STN DBS and CDNF have an additive synergy seen in behavioral tests in a hemiparkinsonian rat model corresponding to late-stage PD. However, these results should be viewed as preliminary and should be tested with other disease models and NTFs. The inefficacy of CDNF alone in an animal model with near complete dopamine depletion suggests that NTFs should be studied earlier in clinical PD. This thesis provides first supportive evidence that one possible solution to overcome the inefficacy of NTFs in clinical trials could be offering NTFs simultaneously with STN DBS, as these therapies seem to have a positive interaction. If nigral injections of NTF are confirmed in future studies to have this interaction, this raises the possibility of providing both NTF therapy and STN DBS through similar neurosurgical approaches.

3) The data show that although electrode location might affect long-term clinical efficiency of STN DBS, the anatomical area of beneficial effect seems to be large. This suggests that some differences in clinical studies of STN DBS might be explained by subtle differences in electrode positioning, which is often omitted from data analysis. Defining the electrode location is also possible using multiple methods, which can improve reliability. The direct visualization of the STN is possible using 1.5T MRI, although indirect methods using midcommissural point and nucleus ruber seem to be more reliable. Electrode locations should be reported in future clinical studies of STN DBS, preferably determined by a combination of different methods. Our data also provide additional support for the effectiveness of STN DBS.

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